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G. C. AINSWORTH and S. D. GARRETT

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DIFFERENTIAL RHIZOSPHERE EFFECTS OF THREE PEA CULTIVARS ON PHYSIOLOGIC RACES OF *FUSARIUM OXYSPORUM* F. *PISI*

By E. W. BUXTON

Rothamsted Experimental Station, Harpenden, Herts.

(With 5 Text-figures)

Pea cultivars Onward, Alaska and Delwiche Commando, differential hosts for three physiologic races of *Fusarium oxysporum* f. *pisi* (cause of pea wilt), exert different effects on the soil microflora. Cultivar Onward, susceptible to race 1, supports more fungi, bacteria and Actinomycetes near its root surface than do either Alaska or Delwiche Commando, which resist race 1. Qualitative differences between rhizosphere populations of the three cultivars are most apparent in *Fusaria* and bacteria.

The germination of spores of the three physiologic races of *F. oxysporum* f. *pisi* was affected by water extracts of the three rhizosphere soils in the same way as earlier with root exudates from the three cultivars. Spores of a race able to wilt a particular cultivar germinate well in rhizosphere soil extract from that cultivar. Germination is decreased, on the other hand, in extracts from the rhizosphere of a resistant cultivar. Spores of race 3, however, were not able to germinate well in rhizosphere extract of cultivar Alaska, which is susceptible to this race.

Soils in which each of the three pea cultivars had grown were inoculated with spores of race 1 and planted with seedlings of the susceptible cultivar Onward. Plants became severely wilted in pots in which Onward had grown and in control pots which had not grown peas, but wilting was less and developed more slowly in pots which had previously been cropped with cultivars Alaska and Delwiche Commando (which resist race 1). It is suggested that substances exuded by roots of cultivars resistant to race 1, together with the effect of altering the soil microflora, prevent race 1 from germinating, thus lowering the amount of effective inoculum and delaying the onset of wilt.

Introduction

Three physiologic races of *Fusarium oxypsorum* f. *pisi* (cause of pea wilt) can be distinguished on the pea cultivars Onward (O), Alaska (A) and Delwiche Commando (DC) (Schreuder, 1951; Buxton, 1955). O is susceptible to all three races, A to races 2 and 3 and DC to race 3 only. The mechanism by which these cultivars differentiate between the three races is unknown, but it may be controlled by root exudates affecting the fungus before it enters the root, that is when it is in the rhizosphere. Buxton (1957) found that root exudates from the three cultivars, O, A and DC, grown in sterile sand, affected germination of spores of the three races differentially. For example, exudate from cultivar A, which resists race 1, inhibited germination of race 1 spores, but enhanced germination of race 2 spores, to which it is susceptible. In normal soil, however, any such specific effects of root exudates would probably be modified by the rhizosphere

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microflora. For instance, production of metabolites by soil organisms might adversely affect the pea wilt Fusaria (Brian, 1949). Competition for substrates, on which survival as saprophytes would be possible, is also intense (Garrett, 1950). Such activities, in conjunction with availability of root exudates, would play an important part in controlling germination and growth of *F. oxysporum* f. *pisi* in pea rhizospheres, and thereby affect the inoculum available for root invasion.

In the present paper an attempt is made to assess the relative importance of the effects of root exudates and of the fluctuating populations of rhizosphere micro-organisms on the pea wilt *Fusaria* when the three differential hosts are growing in unsterile soil.

Peas can become naturally infected with *F. oxysporum* early in their growth, and wilting is usually well developed by the time pods begin to set. These experiments, therefore, covered the first 4 weeks of plant growth, by which time plants normally flower in the glasshouse.

Materials and methods

Pea plants, cultivars O, A and DC, were grown separately in 4 in. pots (three plants per pot) containing a mixture of light loam soil, peat and sand (four parts soil, two sand, one peat), and were left in a heated glasshouse. The pots were placed in shallow trays and watered from below to prevent root exudates and other substances leaching from the soil and to ensure a constant moisture level. At 7-day intervals, sample pots were removed and the plants dug up carefully to avoid damaging the roots. Surplus soil was shaken from the roots, and rhizosphere soil, consisting of small soil crumbs adhering to root surfaces, was removed by immersing the roots of each plant in 10 ml. sterile glass-distilled water contained in 30 ml. (2·5 cm. diam.) specimen tubes. The roots were agitated until no more soil adhered to them, after which root fragments, ten from each plant, each 0·5 cm. long, were washed in tap water for 5 min. and plated on 2 % potato-dextrose agar. The rhizosphere soil suspensions were diluted to 1/1000, and 0·1 ml. of each of the diluted suspensions was plated in 15 ml. Czapek-Dox + yeast extract agar (Warcup, 1950). The pH of this medium was *c.* 5·0, (not 4·0, as recommended by Warcup). Some bacteria grew, though not sufficient to prevent fungi growing on the plates. After 4 days' incubation at 25° C., colonies of soil fungi, bacteria and Actinomycetes were counted and, after a further 4 days, the plates were re-examined to identify the more commonly occurring species. At the same time, suspensions were also prepared of small amounts of soil taken at 2–3 cm. away from the roots in pots which contained pea plants, and from control pots containing soil only. After the pH of soil suspensions had been measured (glass electrode), they were filtered through Whatman no. 1 filter-paper in a Buchner funnel, concentrated to $\frac{1}{2}$ volume *in vacuo* at 40° C. and then Seitz-filtered to remove bacterial contaminants. The filtrates, which presumably contained root exudate, soil extract, growth products of soil rhizosphere organisms and breakdown products of root exudate produced by the action of soil organisms, were then tested to see how they affected the germination of spores of the three *Fusarium* races (Buxton, 1957).

Soil residues from each specimen tube, obtained by filtering the suspensions, were oven-dried and weighed to calculate the number of organisms per g. dry soil. Expressing changes in rhizosphere populations in relation to any changes that might occur in soil away from roots provides the best indication of the relative effects of the rhizosphere environment. For this purpose, the *R/S* indices were calculated, where *R* = the number of rhizosphere micro-organisms per g. dry soil and *S* = the number of micro-organisms per g. soil away from roots (Timonin, 1940).

Results

The rhizosphere of plants usually supports more species and individuals of soil organisms than do micro-habitats elsewhere in the soil. In the experiments reported here, many different species of fungi, bacteria and Actinomycetes were encountered near pea roots, although several of them occurred only rarely. For comparative purposes, the more prevalent species were grouped together under the headings shown in Table 1.

Table 1. *Major taxonomic groups and common species of soil microflora growing on Czapek-Dox + yeast agar from soil dilutions from rhizospheres of pea cultivars Onward, Alaska and Delwiche Commando*

Major groups of soil organisms	Percentage frequency within the group	Common species of fungi	Less common species of fungi
F. oxysporum Fr. (sensu Snyder & Hansen)	33	*Trichoderma viride* Fr.	*Pullularia pullulans* (de Bary) Burkh.
F. solani (Mart.) App. & Wr.	19	*Gliocladium roseum* Corda	*Chaetomium* sp.
F. roseum (sensu Snyder & Hansen)	48	*Pythium* spp.	*Aspergillus niger* van Tiegh.
Mucor spp.	10	—	*Phoma* spp.
Rhizopus spp.	80	—	*Cladosporium herbarum* (Pers.)
Mortierella spp.	10	—	—
Penicillium spp.	—	—	—
Bacteria	—	—	—
Actinomycetes	—	—	—

Root surface microflora

Root surfaces of the three pea cultivars O, A and DC supported growth of many different fungi, mainly Fusaria, Mucorales and Penicillia, with occasional *Pythium* and *Trichoderma* species (Table 2).

The increase of Fusaria on O roots from few at first sampling to many later on, contrasts sharply with their lower frequency on A and DC at all four growth stages. There were more Fusaria on roots of O than on A or DC roots after 21 and 28 days (analysis of variance showed differences significant at 0·01 % level). Mucorales were common at all stages of growth on all three cultivars, Penicillia were rare, and bacteria, although common on all roots examined, were less so on A than on O and DC. Actinomycetes, *Trichoderma* and *Pythium* occurred only rarely, but *Pythium* increased in the later growth stages, especially on DC. Although it is

difficult accurately to assess the numbers of propagules on root surfaces (Harley & Waid, 1955), Table 2 shows clearly that some groups, for example the Fusaria, were more preponderant at certain growth stages of particular cultivars than at others.

Table 2. *Incidence of soil microflora on washed roots of pea cultivars Onward, Alaska and Delwiche Commando at four successive growth stages**

Age of plants (days)	Fusaria	Mucorales	Penicillia	*Trichoderma*	*Pythium*	Bacteria	Actino-mycetes
			Onward				
7	3	45	3	0	0	53	0
14	38	60	3	0	2	62	0
21	55	62	5	0	0	45	0
28	62	60	7	0	3	58	0
			Alaska				
7	30	78	2	0	0	55	0
14	28	47	0	5	5	33	0
21	30	50	5	0	0	33	2
28	35	65	2	5	5	33	2
			Delwiche Commando				
7	25	58	0	5	0	43	0
14	40	56	0	0	0	65	0
21	38	50	10	3	8	55	2
28	20	48	2	0	8	50	0

* Each sample consisted of sixty root fragments from each cultivar. Results are expressed as percentages.

Rhizosphere populations

Numbers of micro-organisms developing on the agar plates (expressed per g. dry soil), colonized from the rhizospheres of the three pea varieties and from soil away from roots, are shown in Fig. 1.

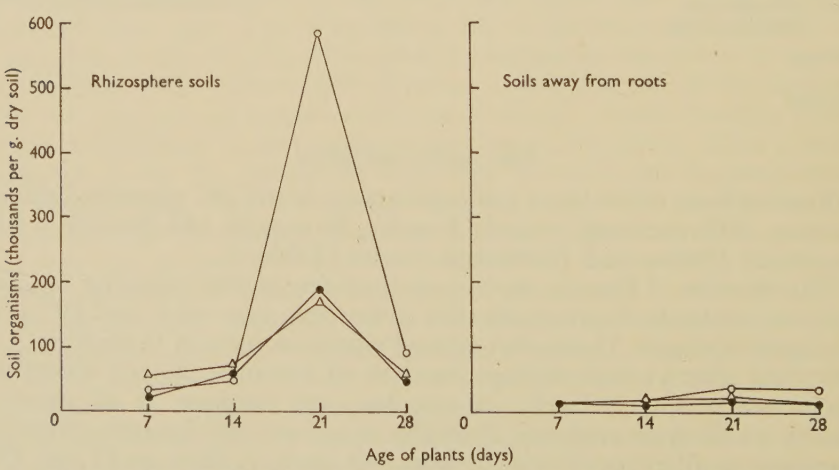

Fig. 1. A comparison of rhizosphere populations of three pea cultivars, differential hosts for three physiologic races of *Fusarium oxysporum* f. *pisi*. Onward, ○; Alaska, △; Delwiche Commando, ●.

The microbial populations of all three rhizospheres increased up to the 21st day of plant growth, when they declined suddenly. O had almost three times as many micro-organisms in its rhizosphere as A or DC after 21 days' growth, although it started with fewer than did A. Such changes might be associated with differences in growth rates of leaves or roots, but Fig. 2, which gives dry weights of the three cultivars, shows that their growth rates increased uniformly within each.

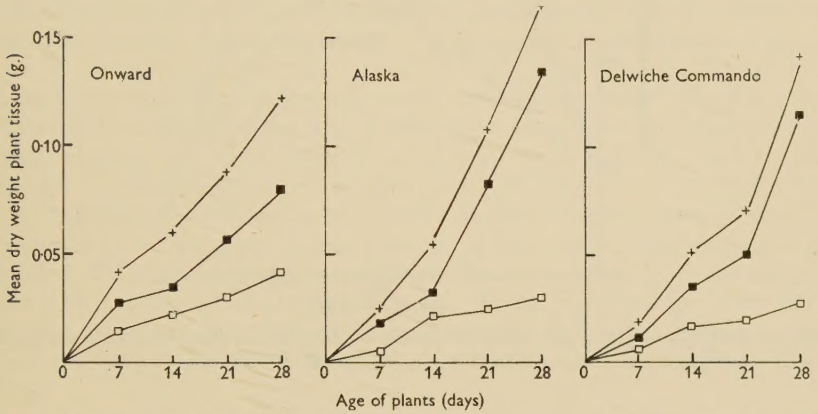

Fig. 2. Growth rates of three pea cultivars, measured as dry weight of tissue produced in unit time. ■, Leaves and stems; □, roots; +, whole plant.

Growth rates of the aerial parts of A and DC were greater than that of O, but there was nothing to indicate why a great increase of rhizosphere organisms should occur 3 weeks after planting. The pH of each rhizosphere soil suspension (Fig. 3) increased to a peak and then reverted towards increased acidity after 28 days. The peaks occurred at 7 days for O, at 14 days for A and over 7–21 days for DC.

Soils away from roots in the pots containing pea plants also increased in pH in the same way. As the microbial populations remained unchanged in soil away from roots (Fig. 1), it seems unreasonable to assume that changes in pH directly cause the changes in rhizosphere populations. It is more likely that the micro-organisms themselves caused the changes in pH, changes which reached beyond the rhizosphere. Root exudates produced in sterile sand at different stages of growth from O, A and DC peas did not significantly change in pH (Buxton, unpublished data).

Qualitative changes in rhizosphere populations

The categories listed in Table 1 were recorded separately on all dilution plates of rhizosphere soils, of soils away from roots and of soils from pots which had grown no peas. The *R/S* indices (Table 3) are calculated from pooled means of replicates from two separate experiments, which gave similar results.

Fusaria rapidly increased in the rhizosphere of O and, later, of A, but decreased in DC rhizosphere. Mucorales progressively established them-

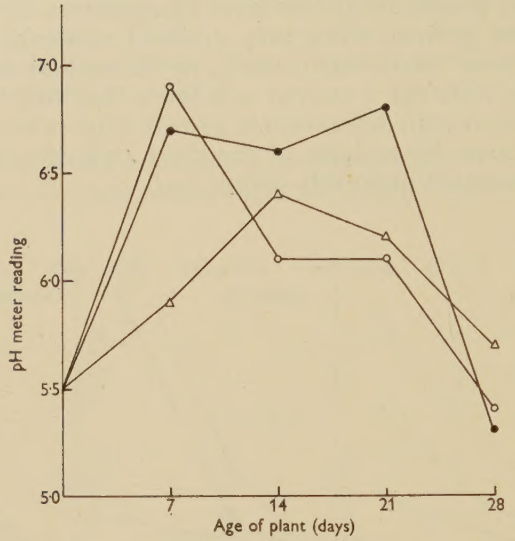

Fig. 3. Changes in pH of soil extract from the rhizospheres of three pea cultivars during 28 days' growth. ○, Onward; △, Alaska; ●, Delwiche Commando.

Table 3. *R/S index numbers for nine groups of micro-organisms in rhizospheres of three pea cultivars Onward (O), Alaska (A) and Delwiche Commando (DC)*

		Age of plants (days)			
		7	14	21	28
Fusaria	O	0·2	48	7·2	32
	A	7·5	8·1	3·8	31
	DC	8·5	4·8	0·7	1·7
Mucorales	O	0·4	1·7	0·3	0·4
	A	0·4	4·0	0·6	0·8
	DC	0·3	3·2	0·4	1·0
Penicillia	O	0	0·9	0·4	0·7
	A	0·8	3·0	0·8	0·3
	DC	12	1·1	2·0	0·9
Bacteria	O	0·3	11	1·2	0·6
	A	1·3	3·1	1·1	2·4
	DC	0·5	4·5	1·7	1·1
Actinomycetes	O	0	0·3	0·5	0
	A	0	0·7	0·4	0
	DC	0	1·3	0	0
Trichoderma	O	0	3·0	0·7	0·5
	A	7·5	4·0	1·0	2·0
	DC	12	1·0	0·7	1·2
Pythium	O	0	0	0	7·0
	A	0	0	0	0
	DC	0	0	0	0·8
Gliocladium	O	13	0·6	0·5	7·0
	A	4·0	0	1·0	0·7
	DC	1·0	0	0·9	3·0
All others	O	0·3	5·1	16	0·5
	A	1·1	73	28	2·0
	DC	0·9	3·6	4·6	0·7

selves in all three rhizospheres up to 14 days, but less so in O, and generally decreased after the 21st and 28th days of growth. Penicillia were abundant at first in DC but decreased after 7 days; they were maximal at 14 days in A, but were never common in O. In contrast, bacteria were much more abundant in O after 14 days than in A or DC, with a peak again reached in all three cultivars after 14 days' growth. Actinomycetes occurred sporadically; they slightly increased in the rhizosphere in DC after 14 days, but were less frequent in other rhizospheres than in soil away from roots. *Trichoderma viride* was more abundant in rhizospheres than in control soils, especially at early stages of growth. In contrast, O rhizosphere supported fewer of this species than did A and DC. *Pythium* was unaffected by the rhizospheres until the last growth stages, when it increased sharply, more in O than in DC. *Gliocladium*, like *Trichoderma*, was more abundant at earlier growth stages, was more evident in O than in A and DC, and was little affected by the rhizosphere of DC until 28 days had elapsed. Other micro-organisms (referred to in Table 1 as less common species) reached a maximum at 21 days on O and DC, at 14 days on A, and then suddenly declined. *Trichoderma*, *Pythium* and Penicillia were generally more abundant when pH was low (Table 3 and Fig. 3). At no growth stage were the *R/S* indices of bacteria as high as those recorded from pea rhizospheres by Adati (1939), who, in one experiment, found an index of 197 for this group.

Effects of rhizosphere soil extracts on physiologic races

Spore germinations of the three *Fusarium* races, when incubated for 5 days at 25° C. in rhizosphere soil extracts, were compared with those in soil from pots which had contained no pea plants. The treatments were replicated twelve times and the experiments were repeated, substantially similar results being obtained on each occasion. Fig. 4 shows the means of all germination percentages obtained.

In O rhizosphere extract, germination of all three races was stimulated at first, race 3 only slightly. Later all were depressed, but races 1 and 3 were slightly stimulated by extract from 28-day-old plants. In A extract, from 7-day-old plants, race 2 germinated better than did races 1 and 3. Later, after depression of all three races in extract from 14-day-old plants, race 2 germinated well, whereas races 1 and 3 remained depressed. DC rhizosphere extract differentiated sharply between the three races, race 3 germinating well at all stages, whereas the germination of races 1 and 2 was depressed after 14 days and did not increase significantly above the non-rhizosphere soil control level at later stages. A not only suppressed the spore germination of race 1, which it resists, but at 14- and 28-day growth stages it also suppressed the germination of race 3 spores. DC, like A, suppressed races 1 and 2 after 14 days but race 3, the only one able to wilt it, grew increasingly well from 14 days onwards.

Similar differentiations between these races by pea root exudates produced *in vitro* were demonstrated by Buxton (1957), but, contrasting with the results in rhizosphere soil extracts, A enhanced race 3 spore germination. In the current experiments in unsterile soils, some effects of the three exudates were no doubt modified by activities of the rhizosphere micro-

flora. For instance, Table 3 shows that Mucorales, bacteria and Actinomycetes reached a maximum at 14 days, that is, when *Fusarium* germinated least well in all three rhizosphere extracts, except for race 1 in O extract. This does not necessarily imply that the metabolic products of these micro-organisms, or their effects on the pea root exudates, directly suppressed spore germination of the three *Fusarium* races. Rhizosphere

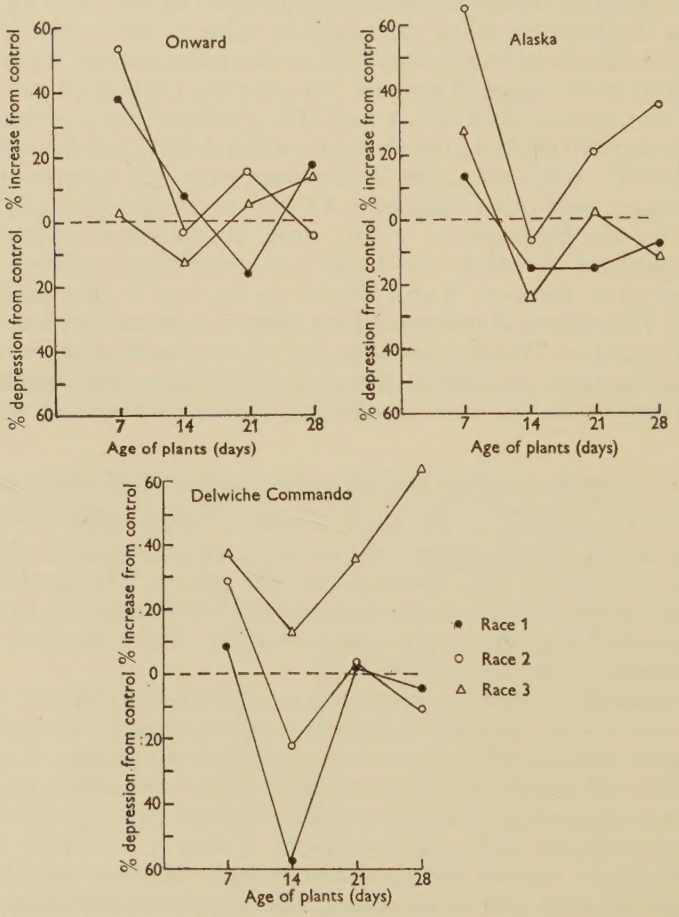

Fig. 4. Effect of rhizosphere soil extracts of three pea cultivars on spore germination of three physiologic races of *Fusarium oxysporum* f. *pisi*.

Fusaria, 33 % of which were *F. oxysporum*, were also abundant at 14 days, but there is no reason to suggest that the three pathogenic races would be stimulated in the same way as were these presumably saprophytic strains.

Residual effects of pre-cropping with resistant peas on wilt caused by race 1

Immediately after each sampling, pots were re-seeded with cultivars O, A or DC; thus each pot supported two successive crops of the same pea cultivar. The ages of the 1st and 2nd sets of plants when harvested were,

therefore, progressively increased or decreased through a period of 28 days. Seven days after the last re-sowing, that is 35 days from the start, all plants and roots were removed, the soil was loosened and aliquots of spore suspensions of race 1 were mixed into the top 2 in. soil in each pot. Twenty-four hours later, 3 in. seedlings of O (susceptible to race 1) were transplanted into the different soils from seed-boxes of unsterile John Innes potting compost.

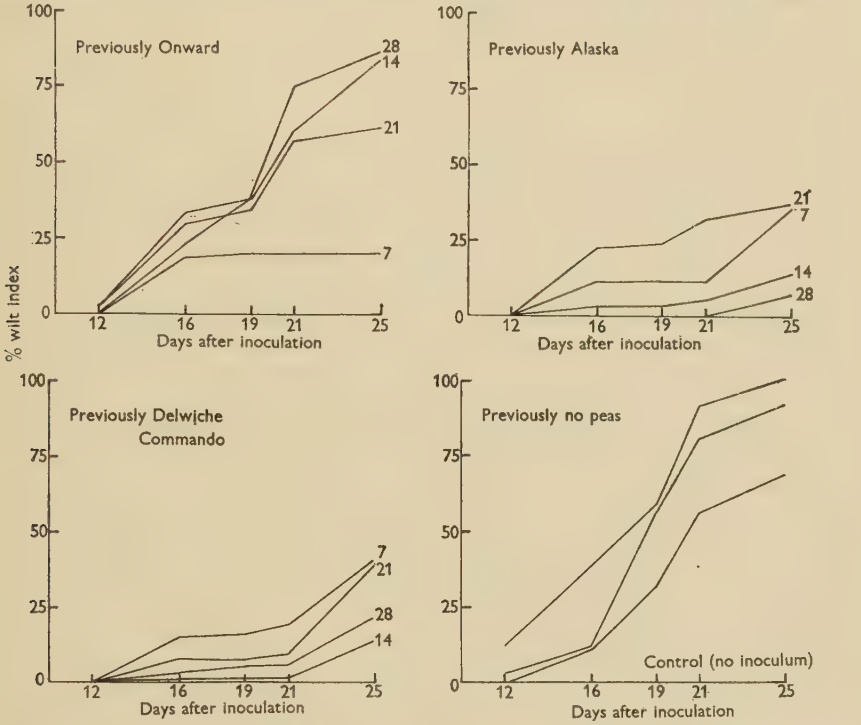

Fig. 5. Progress of wilting in pea cultivar Onward, inoculated with *Fusarium oxysporum* f. *pisi* race 1, when grown in soils previously cropped by Onward (susceptible to race 1), Alaska and Delwiche Commando (both resistant to race 1), and in soil in which no peas had previously been grown.

Wilt symptoms were recorded at intervals, and wilted leaves were expressed as percentage total leaves on each plant. The experiment was twice repeated and similar results were obtained on each occasion. Fig. 5 shows the development of wilt, the figures on each curve referring to the ages of the first set of plants previously occupying the pots, when they were uprooted.

Race 1 caused only a slight and slowly developing wilt in O grown in inoculated soils, where A and DC had previously grown. Typical severe wilting of O occurred in inoculated soils which had previously supported cultivar O or no peas. The suppression of wilt by growing A and DC might result from changes in the populations of various micro-organisms in these soils (Table 3), or from residual effects of inhibitory root exudates (Fig. 4). There is no evidence either way which suggests any antagonism in soil

towards the three *Fusarium* races by the organisms recorded in this work. There is clear evidence, however, of suppression of race 1 spore germination by rhizosphere extracts of A and DC. The time of re-sowing (after the first set of plants was taken out) did not appear to affect the capacity of race 1 to wilt cultivar O, nor is it likely that these results were affected by differences in nutritional level in the soils cropped with O, A or DC, because wilt progressed as quickly in soils which had supported O as in those which had remained fallow.

Discussion

Three facts call for discussion in this investigation of the rhizosphere effects of pea cultivars O, A and DC:

(1) The rhizosphere flora of O, susceptible to race 1, differed quantitatively and qualitatively from those of A and DC, both resistant to this race. The quantitative rhizosphere populations of A and DC, differential hosts for races 2 and 3, did not differ greatly.

(2) Rhizosphere soil extracts of the three cultivars affected spore germination of the three physiologic races of the fungus differently, and these effects were similar to those previously demonstrated with root exudates produced by peas grown in sterile sand.

(3) Soils in which A and DC had previously grown impaired the ability of race 1 to wilt O plants subsequently grown in them.

Because it is difficult to discriminate experimentally between the many factors in the rhizosphere when considering plants under natural conditions, these results have many possible explanations. Obviously the primary influence on a particular soil-borne fungus is exudate from plant roots, which encourages rapid growth and multiplication of micro-organisms. Starkey (1929) found many more organisms in rhizosphere soil than in soil away from roots, and also showed that different species of plants had different effects on the groups of rhizosphere organisms. Starkey's suggestion that such effects may be correlated with characteristics of the plants' physiology, which influence quality and quantity of root excretions, has been partly demonstrated by Eaton & Rigler (1946), who found that micro-organisms on root surfaces of cotton were affected by the carbohydrate content of the roots.

Qualitative effects have been less studied, although Timonin (1940, 1941) showed that some soil fungi, such as *Mucor*, *Cladosporium*, *Penicillium* and *Trichoderma*, were more abundant in rhizospheres of wilt-resistant flax varieties than in those of susceptible varieties. Similar differences in rhizosphere populations occur with pea roots, where, like Timonin's flax varieties, variety Onward, susceptible to race 1, at some stages of growth supports fewer *Mucor*, *Penicillium* and *Trichoderma* near its roots than do varieties A and DC, both resistant to race 1. According to Katznelson & Richardson (1943), considerable differences in nutritional groups of bacteria occur between different rhizosphere populations, groups with simple *in vitro* nutritional needs and those stimulated by amino-acids predominating in the rhizosphere, and those with complex nutritional needs being more prevalent in soil away from roots. This, they considered, was

caused by different types and amounts of amino-acids produced by different root systems. Similarly, Lochhead & Thexton (1947) demonstrated preferential stimulation of rhizosphere bacteria which needed specific amino-acids for growth *in vitro*, but they suggested that other rhizosphere bacteria might affect the amino-acid requirers in other ways. Rovira (1956*a*, *b*) found that the amino-acids in pea root exudates changed with age of the plants, but Buxton (1957) was unable to show that the amino-acid or sugar contents of root exudates from three differential pea hosts, O, A and DC differed from each other. It seems likely, therefore, that the differentiating effects of these three exudates on *Fusarium* physiologic races are caused by other, unidentified, constituents, which may be toxic to certain races, while at the same time promoting growth of others. Moreover the effects on spore germination show clearly that the exudates vary qualitatively with age of the plants. Krassilnikov (1934, 1944), studying wheat root exudates, concluded that *Azotobacter* could not grow in wheat rhizospheres because of the presence of toxins, and striking differences occurred between rhizosphere populations of different crop plants such as alfalfa, clover, wheat, maize, flax and cotton. Meshkov & Khodokova (1954) found that pea and maize root exudates, when produced in sterile culture, differed in their effects on certain soil bacteria. Pea root exudate, for instance, favoured the multiplication of *Azotobacter chroococcum* more than did maize root exudate, and, like pea-wilt Fusaria, the soil bacteria they studied differed in their growth responses to exudate according to the age of the plants.

Although much evidence has been accumulated for beneficial or toxic effects of plant roots on soil micro-organisms, it is technically difficult to differentiate between the various direct effects of the rhizosphere environment on any particular organism, and there exists no known critical method of evaluating the initial or secondary effects of root exudates under natural conditions. With peas, root exudate produced by plants grown in sterile sand (Buxton, 1957) exerted different effects on three physiologic races of *F. oxysporum* f. *pisi*, and similar differences occur with rhizosphere soil extract. Too little is known to assume that root exudates would have similar direct effects on these pathogenic races in nature, irrespective of the effects of microbial activity.

Results of the inoculation experiments described here show that soil can be made inhibitory to a particular race by previously cropping it with a pea cultivar which resists that race, but how long such inhibition persists, and whether or not similar methods might be of value for controlling certain races of the fungus, are problems which need further investigation.

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ON *TRIPOSPERMUM* AND *CAMPYLOSPORA*

By C. T. INGOLD and VIVIENNE J. COX

Birkbeck College, London

(With 2 Text-figures)

The growth of *Tripospermum myrti* in pure culture is reported and the development of the spore is described. This development is shown to agree very closely with that of the spore in *Campylospora chaetocladia.*

In November 1955 a surface scum was examined from a stream at Lullingstone, Kent. The scum had the usual rich spora of aquatic Hyphomycetes, but in addition there were many spores of terrestrial origin, particularly those of *Cladosporium.* Also there were present 5-radiate spores. These were at first thought to belong to an aquatic fungus, but later it was considered much more probable that they had fallen into the stream with the leaves of sycamore and alder which bordered the stream. Later, after leaf-fall was completed, these 5-radiate spores disappeared entirely from the scum. The spores agreed very well with those of *Tripospermum myrti* (Lind) Hughes figured by Hughes (1951). It is to be noted, however, that his figures of *T. myrti* and *T. acerinum* show a slight overlap in the range of both spore size and spore form.

Earlier (May 1955) one of us (C.T.I.), during a visit to Nigeria, had the opportunity of examining spores of *Campylospora chaetocladia* Ranzoni (1953) from the scum of a small stream near Ibadan (Ingold, 1956). When the *Tripospermum* spores were later seen, it was evident that there is very close agreement in spore structure and development between the two genera.

Single spores of *T. myrti* isolated from the Lullingstone scum were planted on nutrient agar and a number of pure cultures were obtained. Growth was very similar on all media tested. Most observations were made on cultures on oatmeal agar. The fungus grows extremely slowly, but without staling. After 5 weeks at 20° C. a colony 1·0 cm. in diameter is produced. It is dense, with low aerial growth less than 1 mm. high, and dark grey, almost black, but with a slight olivaceous tinge. There is no pale colony edge visible to the naked eye. So far as we know, there are no records of any species of *Tripospermum* having been grown in culture. Since cultures sporulate freely, it has been possible to make a thorough study of spore development. Hughes (1951), who examined two British collections of the fungus, 'saw only a few conidia attached to the erect conidiophores and no very young stages'. However, in spite of this he gives an account of spore development which agrees closely with our observations. He says: 'In *T. myrti* the initial elongates somewhat and a septum cuts off an apical cell from a basal stalk cell. The distal cell expands laterally in one direction and a septum arises at right angles to the first one so that a 3-celled

structure in the form of an L is formed. A first and then a second bulge appears in the centre cell of the 3-celled structure and these become cut off by septa; with a further elongation they develop into two divergent conidial arms. The terminal cell (of the original 3-cell structure) in the meantime develops two bulges, one in such a position as to produce an arm parallel with the stalk cell and the other on the opposite side of the cell.' However, in spite of the essential accuracy of Hughes's account, we prefer to present the picture of development in a somewhat different manner. The spore is essentially a lateral branch system of limited growth. The typical mature spore consists of a main septate axis bent forward on itself and of three lateral branches each consisting of 1–4 cells. Not infrequently spores are formed in which one or two of the laterals fail to develop. So far as actual development is concerned the spore arises as a lateral bud from a cell of the mycelium. It can develop from the prostrate mycelium on the surface of the agar, but more usually on an aerial hypha. However, there is no differentiation of definite conidiophores, and the hyphae bearing spores are exactly like those which remain sporeless. Very frequently a number of neighbouring cells of a hypha bear spores. The spore primordium at an early stage is an oval cell constricted where it joins the parent cell and separated from this at a fairly early stage by a cross-wall. As this primordium grows its tip bends over to form a crozier reminiscent of the tip of an ascogenous hypha in the higher Ascomycetes. Either before or after this crozier has formed the primordium becomes 2-celled (Fig. 1) and later a second transverse wall appears, this time in the region of the curvature of the crozier so that it is more or less at right angles to the first cross-wall. By this time the growing tip of the spore primordium is pointing in a downward direction roughly parallel with the basal cell. At this stage the first lateral branch begins to develop from the cell immediately above the basal cell. Shortly afterwards the second lateral develops from the third cell of the primordium, and finally a further arm grows out from the cell which gave rise to the first arm but at an angle of about 80–120° to this arm. The time interval between the origin of the second and third branches may be very small or, perhaps, non-existent. Thus in the mature 5-radiate spore (Fig. 2) two arms (Fig. 2*A*, *B*) are contributed by the main axis and the other three by the lateral branches.

The majority of the spores formed in culture are hyaline, although some are coloured greenish brown by a wall pigment. Most of the hyphae that do not rise above the surface of the agar are hyaline, but the others are pigmented except for the growing hyphal tips. The hyphae are rather broad and mostly short-celled. A feature noticed by Hughes was the presence of short lateral branches resembling phialides. These are also a conspicuous feature of our cultures. Some aerial hyphae are quite thickly beset with these 'pseudophialides'. Each has the form of a typical phialide and the wall is distinctly thinner at its apex. Occasionally something very like a spore is formed at the apex, but this never seems to become detached and if there is any further development the spore-like body simply grows out into a rather narrow hypha. These pseudophialides never appear to grow intermixed with spores along the length of a hyphae. Attempts were made to induce phialospore formation by using different media and by growing

Fig. 1. *Tripospermum myrti.* A–C, mycelium with conidia at various stages of development; D, E, mycelium with phialide-like branches; F, mycelium with inflated terminal cells; G–P, stages in conidium development; Q–W, normal 5-armed conidia; X, Y, abnormal spores. All from pure culture. No contents or pigmentation of cells are indicated.

the fungus under different conditions of temperature and illumination, but these all failed. Another feature of the cultures was the production of spherical terminal cells by certain hyphae. These represent end cells which have become inflated and have no claim to be regarded as spores.

Ranzoni's *Campylospora chaetocladia* was found on submerged decaying leaves (e.g. of *Acer*) from a stream in California. He isolated the fungus in pure culture and also described the process of spore development. His

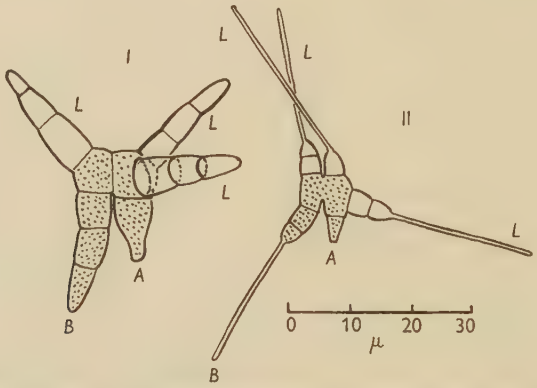

Fig. 2. Comparison of spore of *Tripospermum myrti* (I) from pure culture, and *Campylospora chaetocladia* (II) from a stream at Ibadan, Nigeria. Corresponding parts of the two spores are similarly lettered. The curved main axis (*AB*) of the spore is stippled, the lateral branches (*L*) are shown unstippled.

account agrees very well with that given here for *Tripospermum* and indeed the form of the spore primordium suggested the generic name. The spore consists of a main axis bent forward on itself and of three lateral branches. The essential similarity of structure between the spores in the two genera is shown in Fig. 2, where comparable parts are given similar labels (*B*, apex, *A*, base, *L*, laterals). This raises the question: should *Campylospora chaetocladia* be transferred to the genus *Tripospermum*? At first we were inclined to suggest this transfer. However, because of the very different habitat relations of *C. chaetocladia* (an aquatic fungus) and *Tripospermum* spp. (epiphyllous sooty moulds), because in *C. chaetocladia* the spore arises terminally on an erect conidiophore and not laterally from the parent hypha as in *T. myrti*, and because of the hair-like appendages of the spore of *C. chaetocladia*, it seems best to retain both genera.

In spite of the dangers of phylogenetic theorizing, especially in fungi, it may be permitted to speculate on a possible relationship between the two genera. Perhaps *Campylospora* has arisen from a *Tripospermum*-like ancestor by developing hair-like outgrowth in relation to the aquatic habitat. Clearly an epiphyllous fungus on a deciduous tree must often have the opportunity of being tried out in the aquatic medium. If, as seems very likely, the aquatic Hyphomycetes are derived from terrestrial fungi, no path of migration could be easier than from the branch of a tree to a stream below when the leaves fall in autumn.

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STUDIES ON *PELLICULARIA FILAMENTOSA* (PAT.) ROGERS

III. HOST PENETRATION AND RESISTANCE, AND STRAIN SPECIALIZATION

By N. T. FLENTJE

Waite Agricultural Research Institute, University of Adelaide

(With 2 Text-figures)

The methods of infection of several hosts by a root-attacking strain and a number of stem-attacking strains of *Pellicularia filamentosa* have been studied.

In host-parasite combinations leading to progressive invasion of the host the fungus attaches firmly to the host and grows for some distance along its surface enclosed in a mucilaginous sheath, before forming appressorial structures characteristic of the particular strain. In stem-attacking forms this growth along the surface of the host exclusively follows the lines of junction of the epidermal cells. Penetration by fine infection pegs occurs from the appressoria and invading hyphae rapidly ramify through the host tissue, causing its collapse.

In host-parasite combinations which do not result in progressive invasion, the infection process fails at one or other of four stages: (*a*) hyphae fail to attach to the host surface; (*b*) hyphae attach and grow along the host surface but fail to form appressorial structures or to attempt penetration; (*c*) penetration is attempted but thickening of the cell wall occurs and penetration does not succeed; (*d*) penetration succeeds but the hyphae are killed by a hypersensitive reaction resulting in small necrotic flecks. Failure in the first two stages suggests that chemical stimuli differing with the hosts may be necessary for attachment and penetration by the parasite. The hypersensitive reaction occurs only in certain tissues of a host and the infecting hyphae are killed in the reaction.

Introduction

It is now well established by Storey (1941), Blair (1942), Le Clerg (1941), Richter & Schneider (1953), Flentje & Saksena (1957) that the species *Pellicularia filamentosa* includes a number of pathogenic strains, some limited to hosts of a single family and others capable of attacking wider ranges of hosts. There has hitherto been little comparative study of the initiation of attack by these different fungus strains, to determine the exact method of penetration, and the stage at which specialization or host resistance comes into operation. The preceding paper in this series (Flentje & Saksena, 1957) outlined the pathogenicity of several strains of *P. filamentosa* to various hosts, and the present paper deals with the details of the infection processes for these host-parasite combinations, and with the resistance of the host and the factors governing specialization.

Different workers have studied the infection by *P. filamentosa* of the different parts of plants, e.g. petioles or leaves where there are numerous stomata interrupting the cuticle, stems or hypocotyls where the cuticle is

almost continuous, and roots where there is no cuticular covering. The penetration process apparently varies for these different structures. As demonstrated for other fungi attacking leaves and petioles, Ullstrup (1936) showed that *P. filamentosa* in some cases entered leaves only through the stomata and from there directly invaded underlying cells.

Many strains of *P. filamentosa*, however, invade the hypocotyls directly through the epidermis and not via the stomata. Blackman & Welsford (1916) and later Brown & Harvey (1927) carried out classical studies on this mode of penetration using *Botrytis cinerea* on a number of hosts and on artificial membranes. They established the theory now widely accepted that the hyphae responded to a contact stimulus on the host surface by adhering to the host and forming an appressorium from which a very thin infection peg developed and penetrated the host surface by mechanical pressure. They found no evidence of any cuticle destroying enzyme assisting penetration. Hansford (1945) has shown, however, that *Asterinella entebbeensis* and other related fungi can cause chemical changes in the underlying cuticle without penetration occurring; this phenomenon may be more widespread than is generally recognized. Dickinson (1949*b*), in a study of appressorial formation by rust fungi, showed that on artificial membranes the inclusion of cell fragments in the membrane led to the development of appressoria, penetration of the membrane and formation of substomatal vessicles, whereas this development failed to occur on the same membranes without the addition of cell fragments, suggesting that some stimulus other than purely physical contact was involved. Nakayama (1940), working with *P. filamentosa* on cotton stems, showed that it behaved similarly to *Botrytis*, adhering to the stem particularly in the shallow depressions in the cuticle above the junction of epidermal cells and subsequently forming a special type of appressorium consisting of clumps of hyphae or 'infection cushions'. Direct penetration of the tissue occurred beneath these infection cushions. 'Infection cushions' had previously been described for *Helicobasidium purpureum* by Prillieux (1891) and for *P. filamentosa* on chinese aster by Ullstrup (1936).

Root invasion by *P. filamentosa* can occur in three ways. Nakayama (1940), working with cotton roots, and Kernkamp *et al.* (1952), with cereal and grass roots, recorded direct penetration of epidermal cells and root hairs of cereals and grasses by a mechanism similar to that described above for stems. Cherewick (1940), working with sweet clover roots, and Boosalis (1950), with soybean roots, stated that *P. filamentosa* formed aggregations on the roots which caused discoloration and death of the root cells and penetration occurred subsequently through this dead tissue. Nakayama (1940) and Boosalis (1950) both recorded direct invasion of the roots through natural cracks or wounds.

Behaviour of the root-infecting strain of *Pellicularia filamentosa*

Previous work by Samuel & Garrett (1932), Hynes (1937), Blair (1942) and Flentje & Saksena (1957) had shown that a strain of *P. filamentosa* existed which infected the roots of a wide range of hosts but failed to infect

stems or hypocotyls. The penetration process of this strain was studied using the following two inoculation techniques:

(*a*) Twelve seeds were planted in circles (4–5 cm. diam.) in glass jars of unsterilized soil. When the seedlings were 2–2·5 cm. high, a disk of agar 1·5 cm. diam., cut from the advancing edge of a colony of *P. filamentosa* growing on potato-marmite-dextrose agar, was placed, mycelium down, on the surface of the soil in the centre of the ring of seedlings, pressed down to about 0·5 cm. below the surface and covered with additional soil. Ten replicate pots were sown for each treatment and the seedlings from single jars were removed for examination at daily intervals, beginning on the fourth day.

(*b*) Five per cent of maizemeal-sand culture of the fungus was mixed into unsterilized soil in which seeds were planted in glass jars; the resulting seedlings were removed as described above.

Cabbage, tomato, lettuce and wheat were used as the test hosts. Seedlings were examined: (i) directly under the microscope, unstained; (ii) after staining for 3 min. in cotton blue; (iii) by stripping off selected portions of the epidermis, either with or without previous cotton blue staining, for microscopic examination.

The mode of penetration of roots was the same for all hosts tested. The hyphae grew rather densely around the roots mixing with the root hairs, but not adhering generally to the root surface. Occasionally individual hyphae attached themselves firmly and grew along the axis of the root, adhering to the outside of the cells by a mucilaginous sheath, but not necessarily following the line of junction of epidermal cells, as was described by Nakayama (1940) for cotton stem attack. After growing 1–3 mm. the hypha branched to form a small loosely adhering mat, and penetration by fine infection pegs occurred from the short side branches within this mat which had adhered firmly to the root. Usually more than one hypha combined to initiate the surface mat. Infection usually occurred in the zone immediately ahead of root hair development. Following infection the hyphae spread by penetration via fine infection pegs through the cortical cells, which showed breakdown at or just behind the advancing hyphal tips. The roots usually broke down completely at the area initially invaded to leave a few shortened root stumps; the hyphae did not progress right up the roots into the stem, but left a small root area uninvaded. In lettuce spread subsequent to penetration was much slower, and although there was a collapse of the cortex giving a constricted region, the roots did not break off but appeared to remain functional. Affected roots of lettuce seedlings when placed in a dish of water continued to grow at the tips, whereas affected roots of other plants under the same conditions failed to grow.

On stems of cabbage, tomato and lettuce the root strain showed no reaction, the hyphae merely growing loosely over the surface. On wheat coleoptiles the hyphae attached firmly to the surface and grew up the lines of junction of the epidermal cells, developing short side branches which formed fine infection pegs at their tips. In all cases secondary thickening of the cell walls of the host occurred at the point of attempted penetration and although several hundred examples were examined in detail no hyphae were found to have succeeded in entering the epidermal cells.

Five isolates of the root strain from different sources were examined in detail and all results were identical.

Behaviour of specialized stem-attacking strains

Flentje & Saksena (1957) showed that, in pathogenicity tests with specialized stem-attacking strains, different host/strain combinations resulted in several distinct effects. In some cases there was a progressive invasion of all seedlings of the host (or, in one case, of a percentage of seedlings), in others there was no visible interaction, and in yet other cases there was an intermediate effect, the fungus producing a localized yellow-brown lesion though the host continued more or less normal growth. These interactions were studied separately and in detail, using the same technique as for the original pathogenicity testing, which was described earlier in this paper under method (*a*) for the root strain.

Progressive invasion of all seedlings

Progressive invasion was studied first, as this was regarded as the typical parasitic attack and the three specialized strains crucifer, composite and solanaceous were selected.

(*a*) *Crucifer strain on cabbage, radish, kohl-rabi*; *composite strain on lettuce.* Hyphae growing through the soil contacted the plant stems at a range of angles. Where the contact occurred above the line of junction of the epidermal cells, but not necessarily at right angles to the plane of the stem surface, the hyphal tip frequently attached firmly to the cuticle. There was no sign of swelling of the hyphal tip at the point of attachment, but immediately following attachment staining revealed the presence of a mucilaginous sheath around the hypha. This sheath was not present on the same hypha behind the point of attachment, even after it had attached to the stem; it extended 2–4μ around the hypha and small clay and sand particles frequently adhered to it. Following attachment the hyphae grew up or down the stem directly along the lines of epidermal cell junction, frequently slanting across from one side of a cell to another along the slanting junction between two contiguous cells.

After growing for 1–5 mm. these hyphae sent out side branches at right angles which, after growing for 50–100μ in a straight line curled around to form a tight clump or cushion structure up to 300μ in diameter and 150–170μ high. These cushions were formed in abundance over the stem, from 1 cm. below to 1 cm. above soil-level, within 3–4 days of the first attachment of hyphae to the stems. Hyphal development prior to cushion formation and the appearance of the cushions is illustrated in Fig. 1.

This hyphal organization occurred before any penetration. Adhering hyphae and cushion structures were removed from stems or from epidermal strips by teasing them off with fine glass needles and then lifting them away with fine forceps, and in no case was there any evidence of penetration before the cushion structure was fully developed. Staining of epidermal cells with vital stains such as neutral red revealed no sign of cell disorganization directly below the infection cushions.

Penetration occurred 24–36 hr. after the initiation of the infection cushions in a manner similar to that described by Brown & Harvey (1927) for *Botrytis*. The base of the infection cushion was comprised of slightly flattened hyphal tips opposed at right angles to the plane of the stem surface. Fine infection pegs developed simultaneously from several hyphal tips and penetrated the epidermal cells, regaining normal hyphal diameter immediately they passed through the cell wall. These hyphae grew both inside and between the host cells.

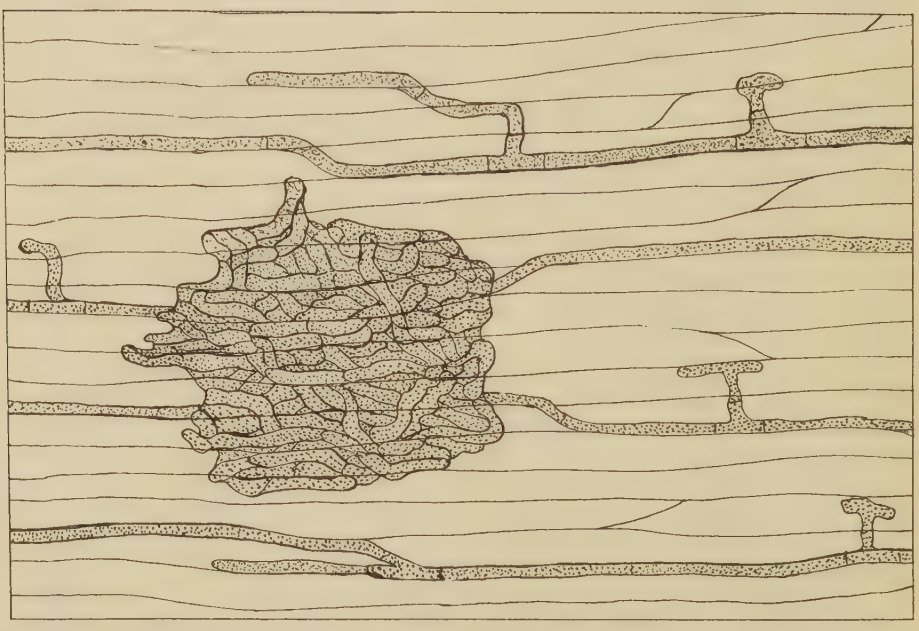

Fig. 1. Growth of *P. filamentosa* hyphae attached to stem and development of infection cushions of the crucifer strain on radish stem. × 200.

Spread subsequent to penetration was rapid. Hyphae penetrated the inside cell walls by the fine infection peg mechanism with no obvious sign of chemical disturbance but were confined almost entirely to the cortical tissue, and showed little extension into the vascular system. Collapse and breakdown of cell contents occurred at or just behind the advancing hyphal tips, the hyphae advancing both upwards into the petioles and leaves and downward into the roots, leading ultimately to complete collapse of the seedlings.

(*b*) *Solanaceous strain on tomato*. The infection progress was identical with that described above except for the detailed structure of the infection cushions. Instead of producing cushion-like structures, the side branches gave rise to short swollen hyphae as shown in Fig. 2, five or six arising very close to each other on the one side branch. These adhered firmly to the stem, and from their tips single infection pegs developed simultaneously to invade the underlying tissue as described above.

At least five different isolates of each of these three strains from varied sources were studied in detail, and within each strain the infection stages were identical.

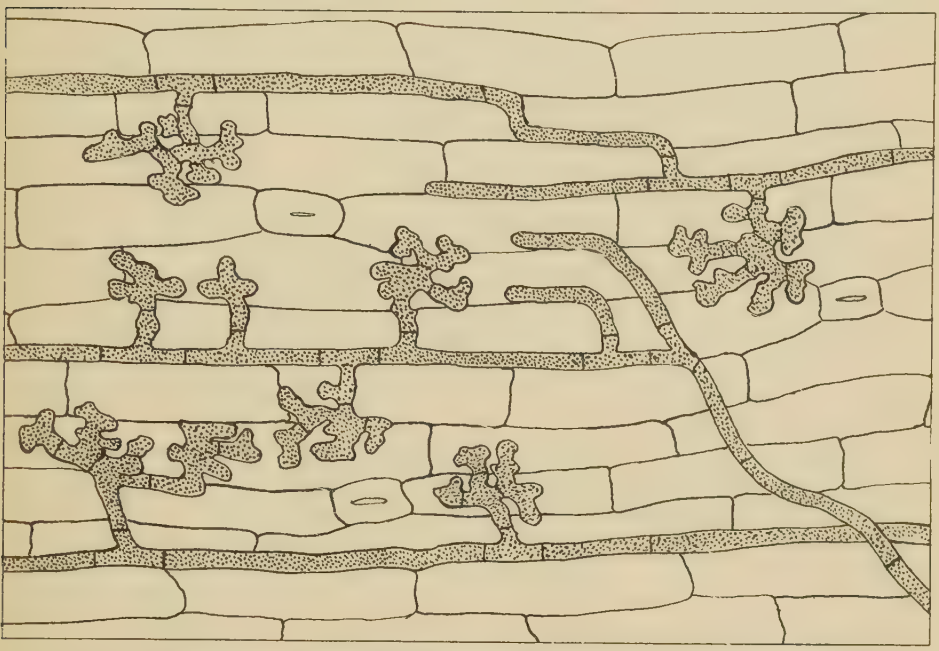

Fig. 2. *P. filamentosa* showing the development of attached hyphae and appressorial structures of the solanaceous strain on tomato stem. × 200.

Progressive invasion of a percentage of seedlings

The composite strain when tested on cabbage consistently produced a progressive invasion of 20–30 % seedlings, the remainder continuing to grow normally. Microscopic examination showed that this strain on cabbage followed the same infection stages as it did on lettuce, but fewer infection cushions developed; many hyphae which attached to the stem failed to form cushions, and the attachment of both the hyphae and cushions to the cuticle was much looser. The mucilaginous sheath surrounding the hyphae appeared to be rather poorly developed in this strain-host combination, and when seedlings were placed in water for examination the cushion structures frequently floated off the stems, a feature not observed with this strain on lettuce stems.

Local necrotic lesions

The crucifer strain (isolates 1, 11, 21, 48, 68; Flentje & Saksena, 1957) when tested on lettuce produced no progressive collapse of the seedling, but localized yellow-brown necrotic spots developed on the stems. Prior to the penetration stage, these isolates reacted to lettuce exactly as they did to

cabbage, penetrating the epidermal cells from infection cushions by fine infection pegs. After passing through the cell wall the infection pegs regained their previous diameter, but instead of ramifying through the epidermal and cortical cells the hyphae appeared to be restricted to the first cell penetrated, or to its immediate neighbours; these cells were killed within a few hours of penetration and turned yellow-brown in colour. The colour developed intensely in the cell walls and appeared to show there first before developing in the actual cell contents. A layer of cells, 1–2 thick, surrounding those invaded were also killed, and failed to take up vital stains such as neutral red; they also showed an intense yellow-brown colour.

The invading hyphae were apparently killed early in this reaction. After 48 hr. they were dissected out from the lesions, but when transferred to agar failed to grow, in contrast to pieces of similar size cut from the hyphae on the outside of the stems, which grew away rapidly when plated on agar.

This reaction appears to be identical with the hypersensitive reaction described by many workers for such obligate parasites as the rusts, and more recently shown to occur with the facultative parasites *Venturia inaequalis* on apple leaves, *Helminthosporium victoriae* on oats and *Phytopththora infestans* on potato.

Isolate 37 (Flentje & Saksena, 1957) of the crucifer strain when tested on lettuce differed from the above crucifer isolates in the prepenetration stages of infection. The majority of hyphae grew over the stem without any noticeable reaction, but occasional hyphal tips on making contact with the stem became slightly swollen, adhered firmly, and penetrated the epidermis directly by means of an infection peg, without growing along the epidermis or forming infection cushions. Following penetration the sequence was the same as in isolates 1, 11, etc., resulting in a hypersensitive reaction, i.e. local necrosis of the host tissue.

No macroscopic reaction

The crucifer and composite strains (five isolates of each) on tomato and the solanaceous strain (five isolates) on lettuce and cabbage produced no macroscopic effect. Microscopic examination of seedlings after inoculation revealed practically no reaction of hyphae on the surface of these hosts. In each case the hyphae grew through the soil and contacted the seedlings normally, but they grew over the surface loosely without adhering or making any attempt at penetration. Occasionally hyphae of the crucifer strain adhered to tomato stems and grew along the line of junction of cell walls, but these never gave rise to infection cushions and no penetration of the host was observed.

Behaviour of less specialized stem-attacking strains

Several isolates, each of which had previously been shown by Flentje & Saksena (1957) to cause progressive invasion in more than two host families, were studied on cabbage, lettuce and tomato seedlings. Progressive invasion of a host was always preceded by the same organization of hyphae on the surface as occurred with the specialized strains, except in the case of tomato seedlings. Each isolate that invaded tomato penetrated

from definite infection cushions, in contrast to the short swollen branches which gave rise to penetration in the specialized solanaceous strain.

Two of the isolates tested progressively invaded cabbage, lettuce and tomato, causing typical 'damping-off' in each host. The remaining three produced typical damping-off in tomato, showed no reaction to cabbage, except that occasional hyphae became attached and grew along the line of cell junction though making no attempt at forming infection cushions or penetrating, but on lettuce developed extensive yellow-brown stem lesions. Microscopic examination of their attack on lettuce showed that the penetration was via infection cushions and identical with that of isolates of the specialized crucifer strain on lettuce. Subsequent to penetration, however, the reaction differed from that of the specialized crucifer isolates, in that although the host cells penetrated were rapidly killed and developed a yellow-brown colour, the hyphae for a time continued slowly to invade new cells, producing a more extensive lesion. Host cells appeared to die several cells in advance of the invading hyphae and even in the vascular tissue, which the fungus failed to penetrate, cells were killed and developed the yellow-brown colour. Ultimately in most cases the invading hyphae ceased growth, the seedlings collapsed at the point of invasion and withered. In a few cases, however, where there were a large number of original points of infection, the invading hyphae after producing small yellow-brown localized lesions grew on rapidly to invade the entire seedling, as in the progressive invasion described above.

Stages in establishment of effective invasion

Summarizing the observations recorded above, it appears that the penetration processes are basically the same for all the strains of *P. filamentosa* tested; direct penetration of the epidermis in roots or of the cuticle in stems occurred with no evidence of any stomatal penetration. Strains varied in certain details and these variations are indicated in the following general outline of the infection processes leading to progressive invasion.

Prepenetration

(1) Hyphal tips became attached to the surface of the tissue and produced a mucilaginous sheath around the tip. Stem-attacking strains attached themselves only above the junction of epidermal cells on the stem.

(2) Attached hyphae changed direction to grow along the surface of the tissue, closely adhering and enclosed by a complete mucilaginous sheath. Stem-attacking strains grew only along the lines of junction of epidermal cells.

(3) Short side branches developed from the attached hyphae.

(4) Side branches gave rise to an appressorial structure. In the root strain this consisted of a small mat of loosely woven hyphae; in the solanaceous stem strain it consisted of four to five separate short swollen branches; in all other stem strains on lettuce, cabbage and tomato it was a tightly woven hemispherical clump of hyphae described as an infection cushion, the base of which consisted of slightly flattened hyphal tips at right

angles to the plane of the epidermis. In all cases the structure adhered strongly to the tissue surface. Up to this stage there was no evidence of attempted penetration or disturbance of either the cuticle or underlying epidermal cells.

Penetration

(5) Penetration followed from Stage 4 by fine infection pegs; in the case of the solanaceous stem strain one peg developed from each short hypha; in all others several pegs developed simultaneously from the hyphal mat or the infection cushion base.

Post penetration

(6) Invading hyphae advanced rapidly through the cortical cells, penetrating cell walls by fine infection pegs. The cells subsequently collapsed, leading to a collapse of roots in the root strain and to a 'damping-off' in young seedlings or a 'wire stem' effect in older seedlings with the stem strains. The conducting tissue showed little or no invasion.

Further observations and discussion

Where no progressive invasion resulted from a host-strain combination, microscopic examination showed that the infection process failed at one or other of four of the stages described above, namely:

Prepenetration

(1) No attachment of the hyphal tip to the surface of the tissue.

(4) No appressorial structures were formed or if formed they were very loosely attached to the tissue surface.

Penetration

(5) Infection pegs failed to penetrate the surface of the tissue.

Post penetration

(6) A hypersensitive reaction occurred immediately after penetration and invading hyphae were killed, resulting in small localized necrotic lesions.

Resistance of the host and strain specialization of the fungus are then dependent on the factors governing these and probably other stages, and some preliminary studies were made of them.

Prepenetration (1). It is suggested from the work of Brown & Harvey (1927) on *Botrytis* and of Dickinson (1949*a*) on several obligate parasites that a contact stimulus causes a germ tube to adhere to the surface of a membrane and controls the direction of germ tube growth. Their supporting evidence was that germ tubes would adhere to, and grow along, a wide variety of plant surfaces and artificial membranes such as gold leaf, collodion and gelatin. In the above experiments, however, *Pellicularia* hyphae showed a marked specificity in their response either to different hosts or to the different tissues, e.g. root and stem, of the one host. The response of six different strains to the roots and stems of four hosts is set out in Table 1.

The root strain showed the most consistent response of any by attaching to the roots of all hosts, but it responded to only one stem structure, that of wheat; all stem strains showed a marked selectivity for the stems of some hosts. It is difficult to reconcile these results with the theory of a stimulus due to purely physical contact, and as a further check the response of these isolates to a series of non-living surfaces including glass, collodion, cellophane and cotton was tested by planting these materials in jars of inoculated soil, using the same method (*a*) as described earlier for seedlings. Seedlings were also grown in these jars at the same time for comparison. In no case was there any characteristic attachment to these non-living surfaces.

Table 1. *Response of six isolates of* P. filamentosa *to contact with the roots and stems of four host plants*

	Lettuce		Cabbage		Tomato		Wheat	
Strain	Stem	Root	Stem	Root	Stem	Root	Stem	Root
Composite	++	o	+	o	o	o	o	o
Crucifer	++	o	++	o	o	o	o	o
Solanaceous	o	o	o	o	++	o	o	o
Less specialized	++	o	o	o	++	o	++	o
Less specialized	+	o	+	o	+	o	o	o
Root	o	++	o	++	o	++	+	++

++, Hyphae adhere and grow along surface in 100 % seedlings; +, hyphae adhere and grow along surface in 20–50 % seedlings; o, no response.

Dickinson (1949*a*) showed that, on any membrane, germ tubes of rusts grew either along the surface or away from it, according to the pre-drying or alcohol treatment of the membrane. He still regarded the stimulus as a contact one as the results were the same both for different fungi and for different types of membranes. Other workers, Nusbaum (1931), Müller (1950) and Stakman (1915) working with air-borne specialized parasites, including *Puccinia* and *Phytophthora*, have also suggested that the stimulus to adhere and penetrate is a simple contact one, as these organisms adhere to and penetrate a wide range of plant surfaces other than those of their appropriate hosts.

The lack of response of *Pellicularia* to non-living surfaces and the selective response of different strains to different hosts indicate that the contact stimulus theory is inadequate to explain the behaviour of this fungus, and it is suggested that a chemical stimulus alone or in combination with a contact stimulus may be important. The mucilaginous sheath developed by *Pellicularia* is absent from the other organisms mentioned above and if this sheath is important in causing the fungus to adhere to the surface, it is possible that a chemical stimulus may be necessary for its consistent development. There is no evidence from any of the above experiments as to whether such a chemical stimulus could be due to the particular molecular configuration of the cuticle surface or to diffusible materials coming through the cuticle from the underlying tissue.

There is no evidence to explain the habit of the stem-attacking strain attaching at, and exclusively following, the lines of junction of epidermal cells when growing along stem structures. Transverse sections of these

stems revealed slight surface depressions along these lines in some hosts but not in others; it is considered unlikely that these depressions as such simply channel the hyphae along these lines. If, however, a chemical stimulus due to diffusible materials influences the growth of attached hyphae, it is possible that the substances responsible may be in higher concentration along these lines, either because the cuticle is thinner here or because the junction of two cells may allow greater diffusion than from one cell alone.

Prepenetration (4). After adhering to the surface of the stem, hyphae always grew along it for a certain distance before forming appressorial structures. These structures were of two different kinds: (*a*) short swollen branches formed by the solanaceous strain; (*b*) infection cushions produced by all other strains. These differences are apparently specific to the fungus strain and independent of the host, as each strain inoculated on a single host formed its characteristic structures. The structure of these two types of appressoria is by comparison with those of *Botrytis* rather complex. In each case a single hypha gives rise to a structure from which a number of infection pegs develop simultaneously. This may be of considerable advantage to the fungus, if a larger number of infecting hyphae more easily overcome host resistance.

The nature of the stimulus leading to the formation of these structures is not clear. The generally accepted hypothesis arising from the work of Brown & Harvey (1927) and others with *Botrytis* is that appressorial formation is due to a contact stimulus, as *Botrytis* freely formed these structures on such surfaces as collodion and gold leaf. In all but a few cases with *Pellicularia*, once the hyphae reacted to a host by adhering, forming a mucilaginous sheath, and growing along the lines of junction of the epidermal cells, these appressoria were formed, and adhered closely to the host, suggesting that the one stimulus might govern both attachment and subsequent appressorial formation. One of the exceptions was the crucifer strain, isolate 37 (Flentje & Saksena, 1957) on lettuce, which penetrated the host immediately the hyphal tip attached to it, whereas on cabbage this isolate formed the usual infection cushions. A second exception was that of the composite strain on cruciferous hosts, where adhering hyphae after growing along the stem either failed to form cushions or formed cushions with very slight or no attachment to the stem. Occasionally other isolates, e.g. the crucifer strain on tomato and less specialized isolates on cabbage, attached to the stems and grew along the lines of cell junction without forming appressoria or attempting to penetrate. These few cases suggest that the stimulus to form appressoria and penetrate the host may be different from that which causes the original attachment of hyphae.

Dickinson's (1949*b*) evidence that species of *Puccinia* only formed appressoria and substomatal vesicles on membranes to which plant residues had been added, and Van Burgh's (1950) demonstration that addition of certain nutrients suppressed appressorial formation support the hypothesis that a chemical stimulus may play some part in the formation of appressoria. For an analogous type of interaction, Raper (1940) showed that a combination of chemical and contact stimuli was necessary for the specialized formation of antheridia and their subsequent penetration of oogonia in *Achlya*.

Previous studies on penetration of hosts by fungi have been carried out almost exclusively with air-borne pathogens, which are transported passively to their hosts and depend on rapid infection for their survival. It is reasonable to suppose that in soil-borne pathogens, which reach their hosts by active growth and are subject to competition from the general soil microflora, the mechanism of penetration may be different. During growth through the soil hyphae must frequently contact the surface of inert material such as sand grains and a selective chemical stimulus for attachment and penetration may be important for survival.

To examine further the possibility of a chemical stimulus both for the original attachment of hyphae and for the formation of appressoria, seedlings of cabbage and tomato were grown to approximately 3 cm. high in seed boxes. They were then removed and a cabbage and a tomato seedling were tied together with cotton as closely as possible without damaging them. These double seedlings were then planted in circles of 5 cm. diameter in jars of soil and inoculated, as described earlier, with either the solanaceous or cruciferous strain of *P. filamentosa*. During the 10 days of this experiment the seedlings increased in diameter and were tightly pressed together over their entire length. There was no evidence from the reaction of the fungal strains of any diffusible chemical moving from one host surface to another, as the two strains organized on and penetrated the stems of their appropriate hosts and showed no reaction on the other seedlings, which remained quite healthy.

A surprising result, however, was the reaction of the solanaceous strain on the petioles of cabbage. These were not touching the tomato seedlings but the fungus reached them by aerial hyphae and organized on them in exactly the same manner as on tomato stems, resulting in a progressive invasion. The invasion ceased abruptly at the junction of the petiole and stem and the latter remained healthy for the duration of the experiment.

One other example similar to this was observed subsequently. Crucifer strain, isolate 37 (Flentje & Saksena, 1957), inoculated into pots of lettuce seedlings penetrated the stems directly at the point of attachment without growing up the stems or forming infection cushions. This penetration resulted in small hypersensitive lesions. In some pots, however, where the lettuce leaves were touching the soil the hyphae grew over the leaves and petioles and formed infection cushions on them in the same manner as on crucifer stems, resulting in a progressive invasion of both leaves and petioles. This reaction was confirmed by a subsequent experiment, in which the soil in the pots was purposely placed in contact with the leaves and petioles, and a similar hyphal organization and progressive invasion was achieved.

The hypothesis that a contact stimulus is solely responsible for the formation of appressoria again seems inadequate to explain the differential reactions of these two strains on different parts of the cuticle on the same plant. If a chemical stimulus is operating, either alone or in conjunction with a contact stimulus, these results indicate that either the same stimulus occurs in different tissues of entirely different hosts or that the one strain can react in the same way to different stimuli, a question which has a very direct bearing on the susceptibility of a wider range of hosts to the less specialized strains.

Penetration (5). In only one host-pathogen combination, namely the root strain on wheat coleoptile, did infection pegs fail to penetrate the cell walls. Numerous infection pegs were developed on each seedling, but in all cases thickening of the cell wall occurred at the points of attempted penetration. There was no apparent disturbance of the cell contents of either the epidermal cells of the host or the surface hyphae of the fungus.

Post penetration (6). Successful penetration of lettuce stems by several isolates failed to result in progressive invasion, and in each a hypersensitive reaction immediately followed penetration; the invaded cells and their immediate neighbours were killed and showed a definite yellow-brown reaction. The invading hyphae in each case had elongated and branched slightly by the time the colour developed, but as they failed to grow when dissected out and (with or without washing) transferred to agar, it was assumed they had been killed by the reaction. As *P. filamentosa* is a facultative parasite, it was unlikely that hyphal death was due to starvation as has been suggested for obligate parasites. As a further check on this, lettuce seedlings were treated with a variety of narcotics as described by Müller & Behr (1949) and then exposed to attack by the same isolates of *P. filamentosa*. In each case the fungus progressively invaded the tissue, with no sign of any hypersensitive effect.

Light intensity is known to affect the hypersensitivity of plants to some obligate parasites and was studied in relation to the isolates of *P. filamentosa* reacting hypersensitively with lettuce. Lettuce seedlings were grown in normal light until the second rough leaf was developed, and then were held at three light intensities, approximately 1500, 1000 and 200 f.c. for periods of 2, 4, 6 and 8 days at 18–20° C. in pots inoculated with the above strains. At 200 f.c. for 4 days or more, progressive invasion of the seedlings occurred, but under all other treatments the host reacted hypersensitively. This is the reverse effect to that generally obtained with rusts, in which restriction of light increases host resistance to infection.

As the invading hyphae were killed in the hypersensitive reaction, and as the reaction also occurred in cells adjacent to those invaded but did not occur in narcotized or recently killed tissue, it was thought that it might be due to a diffusible product of the fungus reacting with intermediate products of the plant to produce some toxic substance. An attempt was made to reproduce the effect by using culture filtrates and actual extracts of the fungus. The isolates were grown in a range of liquid cultures, including decoctions of lettuce, for 10 days; then the mycelium was filtered-off and the filtrate retained. The mycelium was ground up with sand and extracted by heating to 80° C. for 1 hr. Lettuce seedlings grown in soil to the second rough leaf stage were cut off at soil-level and the cut ends placed separately in the above filtrates and mycelial extract. There was no evidence of any hypersensitive reaction after 72 hr. in any of the treatments.

It has already been mentioned that crucifer strain, isolate 37 (Flentje & Saksena, 1957), which produced a hypersensitive reaction on lettuce stems, progressively invaded the leaves and petioles. All other crucifer strain isolates showed the same result when opposed to leaves or petioles instead of to stems. They grew down the petioles rapidly until they reached the junction of the petiole and stem. At this point a hypersensitive reaction

occurred, with all the cells across the base of the petiole and the neighbouring cells of the stem showing an intense yellow-brown discoloration. Apparently all hyphae in this discoloured tissue were killed in the reaction as repeated attempts to culture them by cutting out the tissue and transferring to agar failed to yield any growth. Similar sized pieces of invaded petiole behind the hypersensitive lesion when transferred to agar gave rise to vigorous colonies of *Pellicularia*. Following this hypersensitive reaction there was apparently no penetration of this region by any living hyphae in the petiole, supporting the finding of Müller & Behr (1949) that such hypersensitive areas contain fungistatic or fungicidal substances.

The hypersensitive reaction in the stem, and the absence of it in leaves and petioles, suggests that the plant constituents concerned in the reaction do not occur, or are in too low a concentration to react, in the leaves and petioles. A similar explanation could be offered for the result described earlier, where the solanaceous strain grew down inside the petioles of cabbage, but failed to invade the stems.

The necrotic lesions resulting from the hypersensitive reaction of the less specialized isolates 75, 78 and 79 (Flentje & Saksena, 1957) on lettuce were more extensive than those caused by the crucifer strain. The crucifer strain lesions involved a few epidermal and subepidermal cells which were infected and a surrounding layer one or two cells thick. Isolates 75, 78 and 79 invaded a much greater number of cells and the hypersensitive effect extended to a much thicker layer of non-infected cells, including some of the vascular tissue. This latter effect on lettuce was also obtained by causing an isolate of the crucifer strain to anastomose with one of the composite strain. The cells involved in the fusion were cut out and replated on agar. After the culture had been growing for some time, a single hyphal tip was cut off, grown in culture and again a hyphal tip was removed. The colony resulting from this second hyphal tip was different from either parent in cultural appearance, and when tested on lettuce seedlings it produced at each infection point a much more extensive hypersensitive reaction than that produced by the original crucifer isolate, and exactly similar to that produced by the less specialized isolates. From these results it is suggested that the factors governing progressive invasion, possibly including pectinase secretion, may be distinct from those causing the hypersensitive reaction. When one set of factors is present either progressive invasion or small hypersensitive flecks appear, but when both sets are present the invasion factors modify the hypersensitive reaction by allowing the hyphae to invade further and the toxin causing hypersensitivity to diffuse further out from the hyphal tips.

Although this hypersensitive reaction has been described for at least three other facultative parasites, *Venturia inaequalis* (Nusbaum & Keitt, 1938), *Phytophthora infestans* (Müller, 1950) and *Helminthosporium victoriae* (Paddock, 1953) it has been generally accepted previously as a reaction peculiar to obligate parasites. Its occurrence in interactions of *Pellicularia filamentosa* with different hosts, and the fact that its presence or absence can be arranged by alteration of light intensity, offers many new possibilities for investigating this reaction.

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ANTHRACNOSE OF BANANA (*GLOEOSPORIUM MUSARUM* CKE. & MASSEE) WITH SPECIAL REFERENCE TO LATENT INFECTION IN STORAGE*

By TILOTTAMA CHAKRAVARTY†

Department of Cryptogamic Botany, University of Manchester

(With Plate 11 and 3 Text-figures)

Most of the published works on banana anthracnose caused by *Gloeosporium musarum* Cke. & Massee deal with the infection of harvested and stored fruit. Detailed information regarding the nature of latent infections is inadequate and is the special subject of the present investigation. Cavendish bananas from the Canary Islands and the Gros Michel banana from Jamaica and the Cameroons were available for investigations. By comparing three representative isolations of *G. musarum* from three different localities, it has been shown that comparable strains may be obtained from different localities. By germination experiments in the expressed juice of green and yellow banana skins it has been shown that there is an inhibitory effect of the green skin juice on the germination of conidia. This inhibitory effect may be due to the presence of tannin, which disappears to some extent as the fruit ripens. By artificially inoculating green and yellow bananas it has been shown that *G. musarum* penetrates the cuticle of the host by mechanical means, and in green bananas remains in an inactive condition in the subcuticular region until the fruit ripens, when the fungus resumes activity to produce typical anthracnose lesions. Histological changes caused by the infection are described and discussed.

Introduction

Anthracnose, a disease of the banana fruit caused by *Gloeosporium musarum* Cke. & Massee, is found in all parts of the world where bananas are grown. It was originally described by Cooke and Massee from Brisbane, Australia, in 1873, and was recorded by Halsted, in 1893, on fruit arriving in United States markets. Fruits may be affected in the field, in local markets or during large-scale transport and subsequent marketing.

The most important works on this disease are those of Agati (1922), Toro (1922), Cook & Taubenhaus (1911), Halsted (1893), Wardlaw (1935), and Simmonds (1941). Shear & Wood (1913) have contributed information on the ascigerous stage, their approach being chiefly from the genetical point of view.

Although in some countries *G. musarum* occurs as a parasite on unripe green fruit in the field (Dastur, 1916), its principal activity is on fruit which has been harvested and stored; the published works mostly deal with this aspect.

The most recent investigations are those of Simmonds (1941), whose

* Part of a thesis accepted for the degree of Ph.D. in the University of Manchester.

† Now of Indian Jute Mills Association Research Institute, Calcutta 27.

confirmation of the 'latent infection' theory of Dastur (1916) is of particular interest. By artificially inoculating immature banana fruit in the field, he was able to show that *G. musarum* can remain inactive in a latent condition for a considerable period with subsequent resumption of activity as the fruit ripens. Baker & Wardlaw (1937) detected the presence of fungi within the rind tissue of mature and immature fruits. Wardlaw & McGuire (1931) considered latent infection one of the most important sources of wastage in both Gros Michel and Cavendish varieties, and they have since extended the theory to some other tropical fruits. Baker & Wardlaw (1937) showed by their isolation technique that the latent infections in the fruit exist from an early age; a result later confirmed by Baker (1938).

From a review of the literature on latent infections it is seen that species of *Gloeosporium* and *Colletotrichum* are generally responsible for this type of infection. Other genera such as *Guignardia*, *Phomopsis* and *Botryosphaeria* have also been isolated by previous workers from other young fruits.

A fuller knowledge of latent infection may be of practical importance as ripe rot is one of the major problems in the storage and transport of tropical fruits.

Materials and methods

Cavendish bananas from the Canary Islands and the Gros Michel banana from Jamaica and the Cameroons were available for investigations.

The state of fruits on arrival in Manchester was observed to vary widely. Some consignments were relatively free from disease while others were heavily infected. Occasionally the fruits arrived in a very badly bruised condition as a result of rough handling during shipment. In a heavily infected consignment many different types of rot were present. Fruits were collected from the ripening rooms as required for mycological investigations, soon after their arrival. A wide range of different types of rot was usually available for the isolation of strains of *G. musarum*. For inoculation experiments and for the extraction of the juice of the skin apparently healthy, hard and green fruits were selected. The fungus was isolated from the inside as well as outside the infected fruits or stalks by standard mycological techniques.

Results

Comparison of cultures

Toro (1922) pointed out that biological forms of the anthracnose fungus can be distinguished on the basis of conidial size and cultural characters. Similar attempts were made to compare the strains of *Gloeosporium musarum* isolated from the banana from the Cameroons, Jamaica and Canary Islands during the present investigations.

Representative isolations were grown in Cook's no. II medium* which has been recommended by Toro (1922) as the medium which gives the

* Cook's no. II medium is prepared as follows: to 500 ml. distilled water add 10 g. agar agar, 10 g. dextrose and 5 g. peptone, 0·25 g. dipotassium phosphate and 0·25 g. magnesium sulphate. Boil, filter, tube and autoclave for 20 min. at 15 lb. pressure.

most striking differentiation between the different forms. Observations indicated that collections of strains of *G. musarum* made from different countries may be either comparable or slightly different.

Germination of conidia

Conidia from cultures 2 weeks old were germinated in hanging drops of sterile distilled water in Van Tieghem cells. Under these conditions conidia germinated readily in 4–8 hr. at 24–27° C. During the early stages of germination the conidia swell slightly and often become septate. A germ tube usually grows out from one end of the conidium but septate conidia may produce germ tubes at both ends. Very rarely more than two germ-tubes developed. The germ-tube may be produced from any side of the

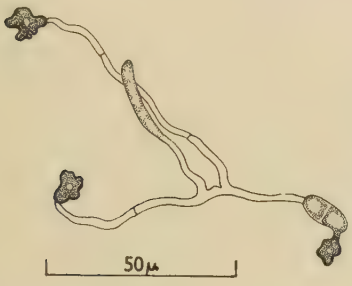


Fig. 1

Fig. 2

Text-figs. 1, 2. A group of germinating conidia showing appressoria with hyaline circular areas; the number of appressoria exceeds the number of conidia.

conidium, but usually it is produced at one end. As the germ tube grows it comes in contact with the surface of the glass and soon its tip swells out into a dark brown, thick-walled spore-like body (Text-fig. 1), the appressorium or adhesion organ. Observations show that it is formed whenever the germ-tube comes in contact with a hard foreign substance. Thus it may be produced soon after the germ-tube is formed so that it lies almost in contact with the conidium; on the other hand, the germ-tube may grow to a considerable length before it is produced. As two germ-tubes may be produced by a conidium, it is quite common for the number of appressoria to exceed the number of germinating conidia (Text-fig. 2). In 2- or 3-day-old hanging-drop cultures, many of the appressoria show distinct hyaline circular areas on the surface in contact with the glass and have a pore-like appearance (Text-figs. 1, 2). More than one of these areas may develop to form infection hyphae. In nutrient media in hanging drops, these outgrowths develop into normal vegetative hyphae.

Germination of conidia in different nutrient solutions and in expressed sap. In this series of experiments conidia were germinated in hanging drops of different nutrient solutions in Van Tieghem cells, and the percentage germinations compared after 4 hr. The following nutrient solutions were used:

Series 1. (*a*) Juice of hard green banana skin. (*b*) Juice of hard green

banana skin and distilled water. (*c*) Juice of hard green banana skin and 2 % malt solution.

Series 2. (*a*) Juice of yellow banana skin. (*b*) Juice of yellow banana skin and distilled water. (*c*) Juice of yellow banana skin and 2 % malt solution.

Series 3. 2 % malt solution only.

Series 4. Distilled water only.

Preparation of the juice. Healthy bananas were selected and washed to get rid of the superficial dirt. The skins were peeled off (avoiding the inner soft part of the skin and the pulp), and minced, the juice being squeezed out and filtered through a sterile Seitz filter.

Table 1. *Percentage germination of conidia of* Gloeosporium musarum

(Duration, 4 hr.; juice used, banana skin (green or yellow).)

Observations (independent)	Without juice		With juice					
			Plain juice		Juice + 2 % malt extract		Juice + distilled water	
	2 % malt extract	Distilled water	Green	Yellow	Green	Yellow	Green	Yellow
1	36	20	13	43	33	64	34	82
2	40	21	8	53	30	63	41	41
3	18	20	6	58	26	52	34	36
4	38	16	10	65	32	46	36	38
5	19	18	8	70	40	48	22	43
6	20	32	15	61	33	36	32	33
7	25	8	16	80	25	41	40	49
8	41	20	20	75	38	58	33	51
9	32	14	16	60	26	33	35	36
10	18	15	17	80	42	74	28	23
Total	287	184	129	645	325	515	335	432
Group total	471		2381					
Grand total	2852							

Table 2. *Analysis of variance*

Source	Degrees of freedom	Sum of squares	Variance	Variance ratio	*P*
Juice	1	3,410·83	3,410·83	34·6	$<0{\cdot}001$
Treatments (T) (gr. I)	1	530·45	530·45	5·4	$0{\cdot}05>P>0{\cdot}01$
Varieties (V) (yellow and green)	1	10,746·67	10,746·67	109·7	$<0{\cdot}0001$
Treatments (gr. II)	2	162·09	81·04	0·82	$>0{\cdot}2$
Interaction (V × T)	2	4,841·58	2,420·79	24·5	$<0{\cdot}001$
Error	72	7,089·00	98·46	—	—
Total	79	26,780·62	—	—	—

The data are set out in Table 1. An analysis of variance in percentage germination in different nutrient solutions was performed according to the standard procedure (Table 2). From this analysis it is seen that:

(1) The presence of juice materially affects the germination of the conidia.

(2) The effects of treatments, viz. germination in presence of (*a*) 2 % malt extract, and (*b*) distilled water, without juice are significant at

5 % level but not at 1 % level. This indicates that the difference in treatment effects, if it exists at all, is not very sharply defined.

(3) The effects of the juice from fruits at different stages of ripeness, viz. green banana skin juice and yellow banana skin juice, are significant at 1 % level—leading to the view that juice of yellow banana skin stimulates germination. Moreover, the juice of hard green banana skin (plain) has actually been deterrent to the growth of conidia. This is ascertained also from comparing the effects of green juice and the treatments in (2). It is probable that the juice of green banana contains some inhibitory substance.

(4) The different treatments with juice, viz. plain juice, juice + 2 % malt extract, juice + distilled water have no significant effect (even at 5 % level) on the germination of conidia.

(5) The effects of interaction between the grades of maturity and the treatments are significant at 1 % level. This is due to the significant difference in the effect of stage of ripeness, bearing out the views in (3) above.

Infection experiments

Experimental procedure and observations. The Cameroon Gros-Michel banana was the first fruit available for infection studies. Healthy, hard, green, immature bananas were selected and washed thoroughly with sterile water after disinfection with 0·1 % mercuric chloride. Conidial suspensions were made up in sterile water. The density of the suspension was such that it had a faint milky appearance to the naked eye.

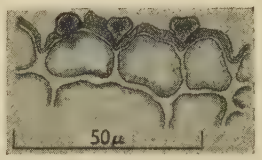
Observations on microtome sections of inoculated tissues are summarized in Table 3 (and see Pl. 11).

Table 3. *Observations on sections of banana tissue inoculated with* Gloeosporium musarum

Material	Observations
A (1)	Germination of conidia, the germ tubes with appressoria in the grooves of epidermal cells; infection hyphae produced from appressoria
A (2)	
A (3)	Penetration of the cuticle with infection hyphae
B (1)	Germination of the conidia; germ tubes have produced appressoria
B (2)	Infection hyphae and penetration present
B (3)	Penetration, subcuticular hyphae, intercellular and intracellular hyphae present
C (1)	Germinating conidia with germ tubes, germ tubes have produced appressoria
C (2)	Infection hyphae and penetration present, intercellular and intracellular hyphae present one layer below
C (3)	Intercellular and intracellular hyphae observed 3 to 5 layers below
C (4)	Formation of acervulus observed; all stages from very young stage with the cuticle of the host tissue still intact, to the mature acervulus showing the cuticle of the host disrupted, and abstriction of conidia observed
A (1), A (2), A (3)	Tissues fixed after 24, 48 and 72 hr. from the inoculated hard green and immature bananas
B (1), B (2), B (3)	Tissues fixed after 24, 48 and 72 hr. from the inoculated less green bananas
C (1), C (2), C (3), C (4)	Tissues fixed after 24, 48, 72 and 116 hr. from inoculated fairly ripe bananas

Penetration of the host. In the bananas used the cuticle was fairly thick and therefore presents difficulties in the study of the details of penetration of the germ tube. Microtome sections were cut 8–10 μ thick and stained with aniline blue in lactophenol followed by Sudan III made up according to the formula used by Dey (1919).

The sections revealed that the germ tube soon forms a brown, thick-walled more or less spherical appressorium closely pressed against the surface of the fruit, the part in actual contact becoming somewhat flattened. Moreover, it shows a tendency to form in the grooves between the epidermal cells, thus giving it a firmer attachment (Text-fig. 3). The appressoria adhere firmly to the cuticle by means of a mucilaginous sheath. Further observations on the formation of these appressoria showed that the epidermal wall becomes slightly distended when the penetration is about to take place, and a mechanical pressure is created which actually breaks the wall. This is preliminary to penetration. No evidence of the cuticle being softened or dissolved was found. The next step is the penetration of the cuticle. When the resistance offered by this layer is overcome, the infection hypha sometimes swells at the tip within the host cell (Pl. 11, figs. 1, 2). This was observed only in two sections. The infection hypha from the appressorium typically passed through the epidermal cells, but one or two examples of penetration by way of the stomata have also been observed.



Text-fig. 3. Vertical section through inoculated tissue showing the formation of appressoria in the grooves of epidermal cells.

It will be seen from Table 3 that in hard green immature bananas the penetration of the cuticle by infection hyphae was observed after 72 hr. (A–3), whereas in the less green fruit the penetration was observed after 48 hr. (B–2, C–2). There was no evidence of penetration of the cuticle in any of the materials fixed after 24 hr.; these revealed the germination of conidia and production of germ-tubes and appressoria only.

Histological changes caused by the pathogen with special reference to the latent infection. The fungus appeared to penetrate the cuticle of the host directly, but, in very hard, immature green fruit, it remained in an inactive state in a subcuticular position until ripening of the fruit began. The fungus then spread into the tissues and caused the characteristic superficial lesion. Sections of inoculated green bananas showed that the infection hyphae had not invaded the cellulose portion of the epidermal wall but remained as knots or threads of mycelium lying between the cuticle and the cellulose layers.

After the cuticle has been penetrated, the tissue beneath may slowly become disorganized. Usually the contents of the cell immediately below are disorganized. This commences as a slight browning of the cellulose elements which is followed by the darkening of the adjacent cells. The fungus traverses the tissues of the skin by way of the middle lamellae and intercellular spaces (Pl. 11, fig. 3). In a few cases, the infection hypha was found to be broad and swollen. In advanced stages of infection, the discoloration and collapse of tissues take place at the point of infection, which

becomes dark. The microscopic details are difficult to demonstrate satisfactorily. Intercellular and intracellular hyphae were observed at the depth of one to five layers of cells in the ripe fruits.

Development of acervuli. The development of the acervulus was observed in materials from the inoculation experiments after 116 hr. Most of the acervuli were found to be formed immediately below appressoria and in the subcuticular region (Pl. 11, figs. 4, 5). The tissues beneath the acervuli collapse and become dark; this makes it difficult to observe the advance of the fungus in the host tissue. Acervuli were not observed in sections of hard green bananas. Similar observations on the formation of acervuli on the skin of the fruit were made by Wardlaw (1935) and Simmonds (1941).

Discussion

Although anthracnose of banana, caused by *G. musarum*, is more in evidence in the ripe fruit in storage, infection occurs during flowering and the formation of the young fruit. Such latent infections of immature fruits are well known in a number of tropical fruits. These early infections remain inactive until a precise stage in the ripening process has been attained. Wardlaw, Leonard & Barnell (1939) have shown that the development of *Gloeosporium* skin lesions in the banana and other fruits can be precisely predicted from the trend of the respiration curve. Dastur (1916) showed that occasionally in India these early infections may give rise to the disease in the field while the fruits are still green and immature. More generally, however, as indicated above, the lesions do not appear until a late stage in ripening.

The inoculation experiments on green fruits carried out by the writer revealed that although the infection hypha of the fungus penetrates the epidermal wall, it is unable to exploit the host tissue until fruit ripening is well advanced. During this period the fungus remains inactive in the form of a very short subcuticular hypha. Similar observations were made by Simmonds (1941) for the banana, papaw and mango. According to him, it is as subcuticular hyphae that the fungus persists in the inactive state throughout the latent period. He observed that as the fruit ripens the mycelium appears to be at first intercellular, later becoming both inter- and intracellular. These observations suggest a relationship between the activity of the pathogen and cellular changes in the host tissue. The presence of the fungus in a latent condition in green fruits probably indicates that the fungus enters the fruit as a result of contact stimulus but is inhibited as soon as it comes into contact with cell solutions. This latter view is to some extent borne out by conidium germination experiments in expressed sap of the skin of the green and ripe fruits. The percentage germination in the juice of green fruit skin was much smaller than in the juice of ripe fruit skin, indicating the presence of an inhibitory substance in the green skin juice. Moreover, the juice of ripe fruit skin may have some stimulating effect on the germination of conidia.

Previous workers (Wardlaw & McGuire, 1931; Simmonds, 1941; Barnell & Barnell, 1945) have mentioned the presence of tannin compounds in the tissues of green banana. These compounds disappear during meta-

bolism as the fruit ripens. Barnell & Barnell (1945) have demonstrated the presence of tannin in the latex vessels of the skin and pulp of bananas. As ripening progresses the contents of the latex vessels undergo changes and eventually become caked and brittle. Tannin gradually disappears from these dried latex contents, in the pulp first and subsequently in the skin. They have also mentioned that as the banana ripens its pulp loses its astringent property, this loss being probably associated with the observed changes in the tannin contents of the laticiferous vessels. In their experiments on the determination of the ability of the 'active' tannin to inhibit the activity of a diastase, they observed that an aqueous extract of banana pulp or skin darkens to a brownish hue on standing and this is accompanied by a decrease in the inhibitory activity of the extract towards diastase.

It may be concluded that the inhibitory power of such a solution (expressed juice) is a measure of its active tannin content. These workers have shown that unripe bananas contain a considerable amount of easily demonstrable tannins which do not completely disappear on ripening. As ripening progresses the tannin content of both pulp and skin continues to fall until the eating ripe stage, after which an approximately steady concentration is maintained. In connexion with latent infections, Barnell & Barnell have suggested the existence of a causal relationship between the tannin concentration of the skin and the activity of these infections. Cook & Taubenhaus (1911) also showed that *G. musarum* is to some extent resistant to tannin. Conidia showed complete germination on 0·6% tannin in 24 hr., but would not germinate in media containing higher concentrations. They also observed that the growth of some fungi is at first retarded by certain percentages of tannin and later becomes as good or more luxuriant than on the medium without tannin. Growth is sometimes stimulated by low percentages of tannin; sporulation, for example, was frequently best on media containing a low percentage of tannin. This may explain the occurrence of conidial masses of *G. musarum* on the skin of ripe banana fruit when the tannin content is low but has not completely disappeared.

The author wishes to thank Prof. C. W. Wardlaw for his helpful suggestions and criticism during the progress of this work, Messrs Elders and Fyffes Ltd for kindly granting access to their storage rooms and for supplies of fruit; and Mr E. Ashby for photography.

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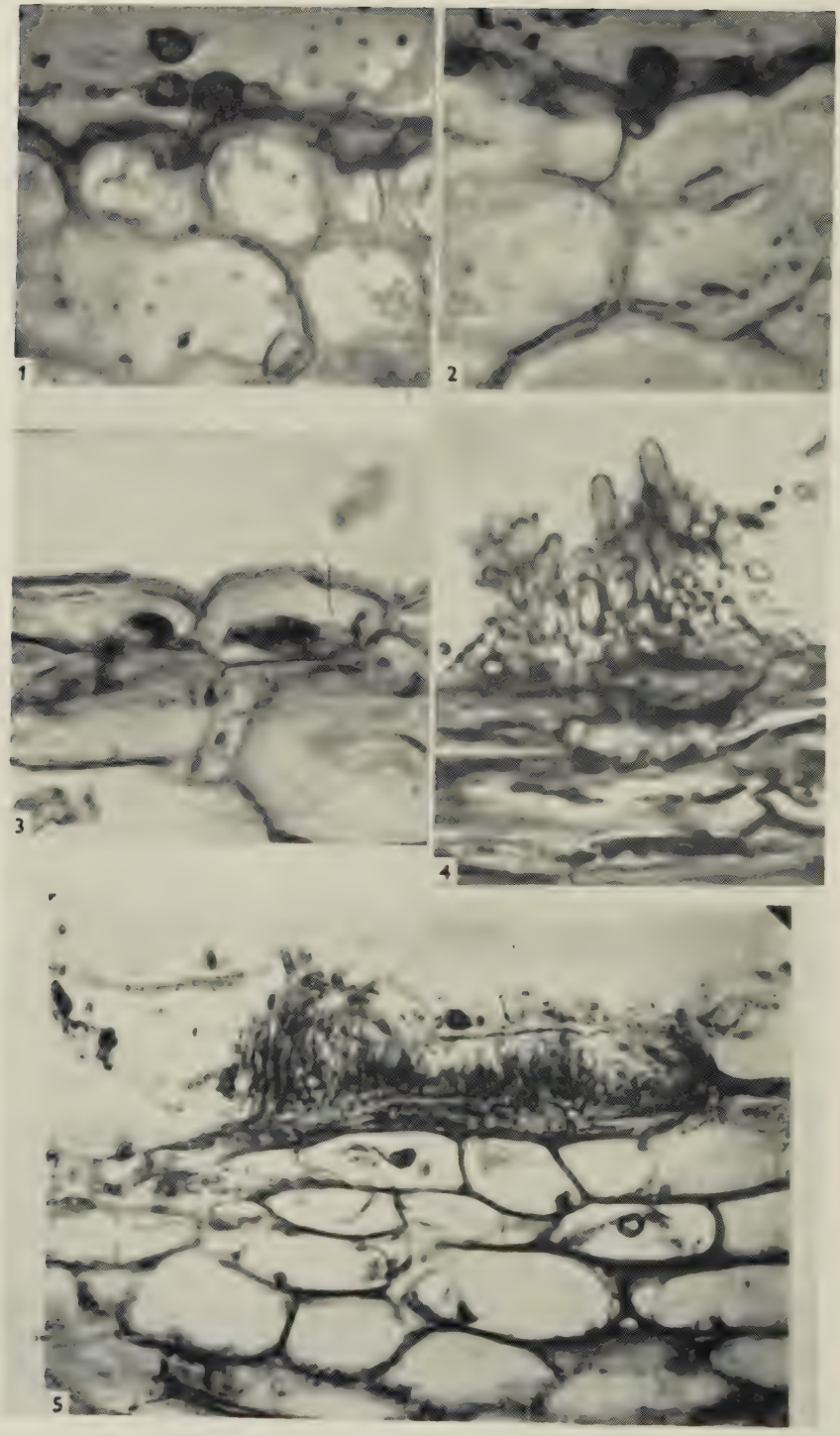

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EXPLANATION OF PLATE 11

Gloeosporium musarum on banana

Fig. 1. Vertical section through inoculated tissue showing penetration of cuticle by the infection hypha (× 1000).
Fig. 2. Vertical section through inoculated tissue showing an appressorium and swollen tip of infection hypha (× 1000).
Fig. 3. Vertical section through inoculated tissue showing intercellular hyphae (× 1000).
Fig. 4. Vertical section through an acervulus showing abstriction of conidia (× 1000).
Fig. 5. Vertical section through inoculated tissue showing the formation of acervulus under the cuticle (× 500).

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DUNG AS A SOURCE OF PREDACIOUS FUNGI

By A. J. JUNIPER

Biological Laboratories, The Polytechnic, Regent Street, London

A brief account of the predacious fungi that have been recorded from different animal dungs.

Historical

Zopf (1888) was the first to describe the capture of a nematode worm by means of a special trap produced by a fungus. He obtained *Arthrobotrys oligospora* Fresenius from horse and rabbit dungs, and gives an excellent illustrated account of the three-dimensional networks that this fungus produces, the way in which the worms are held, and the appropriation of the captive's viscera by endozoic hyphae. Zopf also obtained *Harposporium anguillulae* Lohde from horse dung. Dangeard (1903) described *Protascus subuliformis*, also from horse dung, which attacks free-living nematode worms. More recently, Duddington (1951) recorded nine predacious species from dung.

Methods

Samples of animal dung were collected in clean, 2 oz.-size, tobacco tins and were plated out as soon as possible, as it was found that otherwise moulds tended to overgrow and obscure the slower growing predacious fungi. The agar medium was prepared by placing 10 g. of coarse ground maize-meal in a round-bottomed flask with 1300 ml. of tap water, bringing it slowly to the boil and allowing it to simmer for about 10 min. After cooling, a litre of the liquid was decanted off and filtered through glass wool. 500 ml. of this maize-meal liquor was placed in a litre beaker with 10 g. of powdered agar, warmed over a water-bath and stirred until homogenous. The medium was then dispensed in 350 ml. quantities in 500 ml. conical flasks, and sterilized at 15 lb. for 20 min.

Small pieces of the dung sample were used as inoculum on plates of the maize-meal agar, at least three plates being prepared from each collection. The plates were examined weekly for predacious fungi, and were kept under observation for at least 3 months.

Results

I examined 136 collections of dung and predacious fungi were recorded from fifty-seven. Frequently a heavy fungal growth developed on the plates, making observation of the slow growing predacious forms impossible, and this may account for the lack of records in some samples. Bacterial contamination frequently made it difficult to see haustoria of

the Zoopagales. Table 1 shows the predacious species that have been found and the number of occasions on which each was recorded.

The records suggest that the relatively abundant species are the same from dung as from other habitats (Duddington, 1951, 1954). This is to be expected, as the predacious fungus flora of the dung probably enters from

Table 1. *Predacious fungi found in dung*

Fungus	No. of times recorded	Fungus	No. of times recorded
Acaulopage acanthospora Drechs. var. *magna* Juniper	2	*Acrostalagmus gonioides* Drechs.	2
A. baculispora Drechs.	1	*A. obovatus* Drechs.	11
A. lophospora Drechs.	1	*Arthrobotrys conoides* Drechs.	5
A. rhicnospora Drechs.	1	*A. dactyloides* Drechs.	1
A. stenospora Drechs.	1	*A. oligospora* Fresen.	4
A. tetraceros Drechs.	13	*A. robusta* Duddington	17
Cochlonema megalosomum Drechs.	3	*Dactylaria pyriformis* Juniper	4
Stylopage araea Drechs. var. *magna* Peach & Juniper	2	*D. thaumasia* Drechs.	3
S. lepte Drechs.	2	*Dactylella doedycoides* Drechs.	4
S. rhabdoides Drechs.	2	*D. ellipsospora* (Preuss) Grove var. *prolifera* Dixon var. nov. ined.	1
S. rhabdospora Drechs.	4	*Harposporium anguillulae* Lohde	9
S. rhynchospora Drechs.	10	*H. oxycoracum* Drechs.	8
Zoopage thamnospira Drechs.	1	*Meria coniospora* Drechs.	8
Haptoglossa heterospora Drechs.	2	*Nematoctonus leiosporus* Drechs.	1
Protascus subuliformis Dang.	3	*Trichothecium cystosporium* Duddington	3
		Total number of records	129

Table 2. *Number of predacious fungi from mammalian dungs*

	Elk	Cow	Pony	Horse	Rabbit	Sheep	Hare
No. of collections examined	1	20	6	6	9	4	1
No. of collections from which predacious species were observed	1	8	4	1	5	0	0
Fungus							
Acaulopage acanthospora var. *magna*	.	2	.	.	.	.	.
A. baculispora	.	.	1	.	.	.	.
A. stenospora	.	1	.	.	.	.	.
A. tetraceros	.	3	2	.	1	.	.
Cochlonema megalosomum	.	1	.	.	.	.	.
Stylopage araea var. *magna*	.	1	.	.	.	.	.
S. lepte	.	1	.	1	.	.	.
S. rhabdoides	.	1	.	.	.	.	.
S. rhabdospora	.	1	.	1	1	.	.
S. rhynchospora	.	3	1	.	1	.	.
Acrostalagmus gonioides	.	2	.	.	.	.	.
A. obovatus	.	1	.	.	2	.	.
Arthrobotrys conoides	.	1	.	.	.	.	.
A. oligospora	.	1	.	.	.	.	.
A. robusta	.	2	2	.	1	.	.
Dactylaria thaumasia	.	.	.	.	1	.	.
Dactylella doedycoides	.	.	1	.	.	.	.
Harposporium anguillulae	.	1	.	.	1	.	.
H. oxycoracum	1	.	2	.	.	.	.
Totals	1	22	9	2	8	0	0

the soil. It is interesting to note that Duddington (1954) found that *Arthrobotrys oligospora* was more common than *A. robusta* in soil and I have found the reverse to be true for dung.

Table 2 shows the predacious fungi that have been recorded from the dungs of different mammals. Cow dung has produced more records than the other types, but more collections of cow dung were made than of any other animal. A noteworthy feature is the absence of predacious species from sheep dung, although the samples always contained numerous nematode worms.

Discussion

Dung seems to be a good source of predacious fungi. The condition of the dung seems to affect the number of predacious forms recorded; if fresh, few or no predacious species were recorded. On the other hand, old dung which had been in contact with the soil for some time usually had a rich predacious fungus flora. It therefore seems to me that these fungi enter the dung from the soil. In samples taken from a dung heap at Nocton Fen, Lincolnshire, over a period of a year, it was found that the predacious forms recorded varied from collection to collection. This may be because new forms were continually entering the dung heap from the soil. The water content of the dung seems to be important as I found that most predacious forms occur in moist dung and few or none in dry dung. This is to be expected, as the spores of most predacious species have thin walls. Freezing does not kill all the predacious fungi, as I have recorded them from dung which was frozen solid when it was collected.

I would like to thank Dr C. L. Duddington for his continued help and advice. The material here recorded has formed part of a thesis accepted for the Ph.D. degree of the University of London.

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PHYTOPHTHORA CITRICOLA SAWADA (SYN. *P. CACTORUM* VAR. *APPLANATA* CHESTER)

By GRACE M. WATERHOUSE

Commonwealth Mycological Institute, Kew

(With Plate 12 and 3 Text-figures)

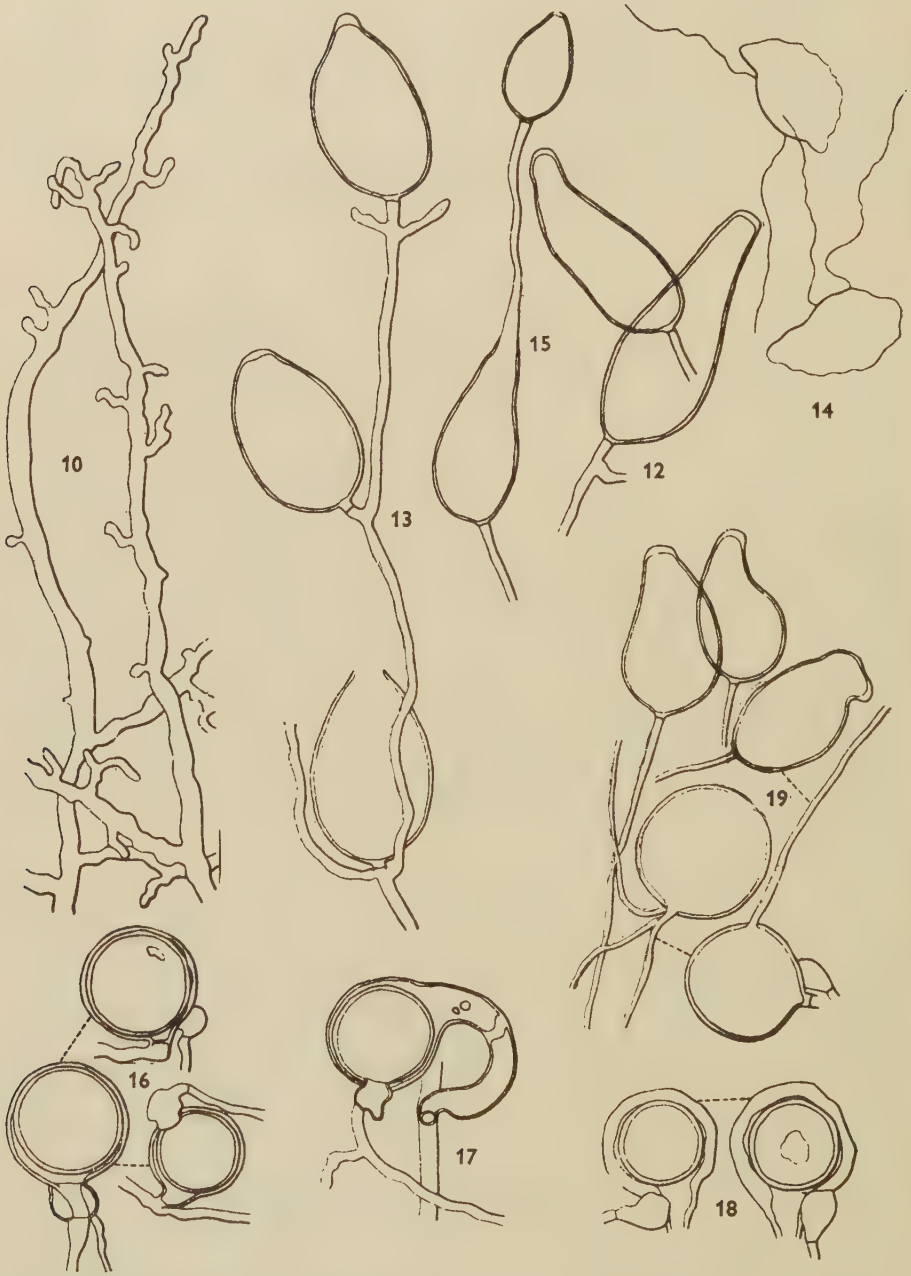
Evidence is presented that *Phytophthora cactorum* var. *applanata* Chester is synonymous with *P. citricola* Sawada, which has priority. A full description of the species is given and a comparison made with *P. cactorum* (Leb. & Cohn) Schroet. It is concluded that *P. citricola* is sufficiently distinct from *P. cactorum* to be retained as a separate species.

Sawada (1927) gave the first description (a full account in Japanese) of *Phytophthora citricola* isolated from brown rot of Sekkan oranges in Formosa. As the account did not include a formal diagnosis the following summary is given of the salient characters, compiled from a Latin diagnosis prepared from the original description by Ito &Tokunaga (1935).

Hyphae 4–10 μ wide; *sporangiophores* simple or sympodially branched; *sporangia* obovoid or obclavate, 21–70 × 15–39 (mean 43·3 × 25·30) μ, with a rounded base and flat, wide papilla; *zoospores* 13 × 8–9 μ; *oogonia* spherical (sometimes attenuated to a funnel-shaped base), hyaline or brown, 21–44 μ in diam., with a smooth wall 1–1·5 μ or rarely 4 μ thick; *antheridia* obovoid, paragynous, rarely amphigynous, 12–13 × 8–10 μ; and *oospores* spherical, almost plerotic, hyaline or yellow, 18–38 μ in diam., with a smooth epispore 1–2 μ thick.

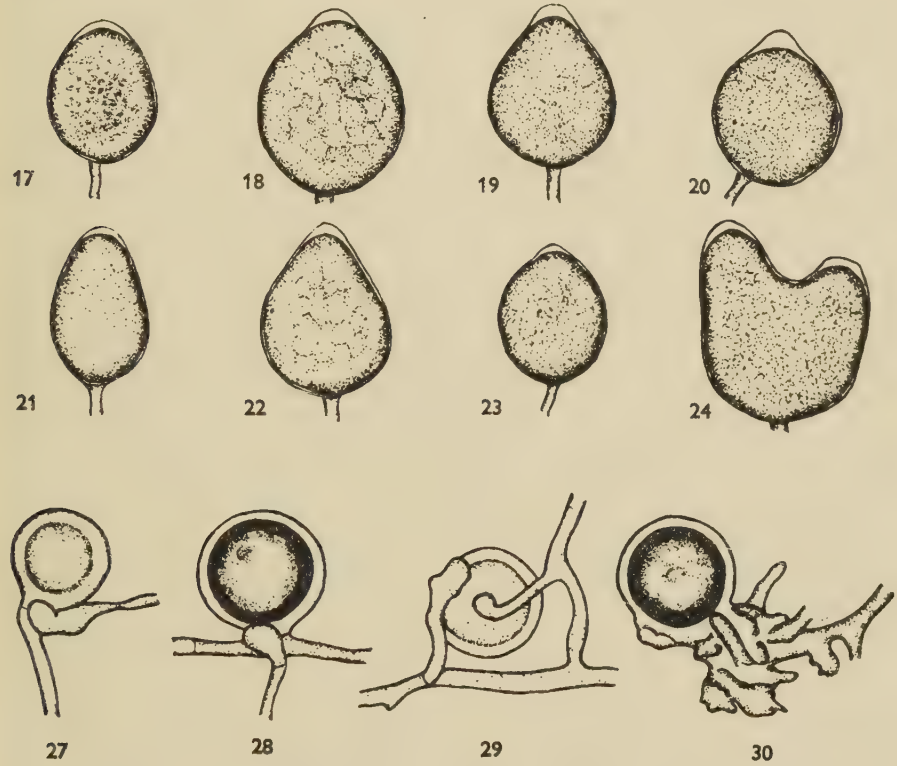
Sawada thought his fungus to be near *P. syringae* (Kleb.) Kleb.

The interesting feature is the flat, wide papilla. It is evident from Sawada's description and figures (Text-fig. 1) that although he used the word papilla he meant that the apex of the sporangium was not conspicuously protruding and the apical thickening was shallow and wide. Tucker (1931) included *P. citricola* as a synonym of *P. cactorum* (Leb. & Cohn) Schroet. He studied a culture sent to him by Leonian, cited as having been isolated by Sawada from *Citrus* sp. in Formosa. Tucker stated that the abundant oogonia, with paragynous antheridia, and oospores were indistinguishable from those of *P. cactorum*. He continued: 'Sporangia were developed rather sparsely and the papillae were not very evident'. This last statement is important because the apex of the sporangium of *P. cactorum* is characteristically papillate—the hemispherical papilla is always quite conspicuous. It suggests that the fungus was not typical *P. cactorum*. The strain of *P. citricola* that Tucker examined was also said to differ from typical *P. cactorum* in being non-pathogenic to potato tubers, but to resemble it in growth characters and temperature relations. Tucker gave 34·3 × 25·6 (extremes 31–37 × 23–27) μ as the sporangial dimensions,



Text-fig. 1. *Phytophthora citricola*. Figures taken from Sawada; 10, 12, 13, 15–19, × 405; 14, × 940

in contrast to Sawada's rather higher figures. His average for ten strains of *P. cactorum*, which from the various features described all appear to me to be typical of the species, was $30{\cdot}39 \times 22{\cdot}77\,\mu$. It is noteworthy that Tucker detected chlamydospores rarely (on oatmeal agar only) or never in *P. citricola* cultures and that sporangia were sparingly produced on solid media.



Text-fig. 2. *Phytophthora cactorum* var. *applanata*. Figures taken from Chester; 17–24, × 1000; 27–30, × 500.

Chester (1932), as a result of comparative studies of *P. cactorum*, *P. syringae*, and a third fungus isolated from diseased lilac in the Arnold Arboretum, named the last (Type A) *P. cactorum* var. *applanata* on the grounds that it differed 'from the species in the consistent presence of flattened, non-prominent papillae'; this was his diagnosis; his drawings are reproduced in Text-fig. 2. In the text he wrote: 'The papillae of Type A are so wholly distinct from those of *P. cactorum* that there is no danger of confusing the two even with a cursory examination of the sporangia, and in the many conditions of sporangium production observed, the character of papilla remained constant'. Another distinguishing feature cited by Chester was the non-caducous nature of the sporangia of Type A; very few were dislodged when water cultures were shaken vigorously, whereas

those of *P. cactorum* were easily detached, each with a very short stump of sporangiophore. He also noticed the presence of a 'granular aura' surrounding the 'triple wall enclosing the oospore' of Type A, in contrast to the smooth wall of the *P. cactorum* oospore. In Chester's discussion of the systematic position of *P. c.* var. *applanata* the possible relationship with *P. citricola* was not considered; moreover, the latter name was omitted, unfortunately, from the list of synonyms of *P. cactorum* given in the paper, though the list was apparently taken from Tucker's monograph.

Leonian (1934) agreed that *P. c.* var. *applanata* was 'readily separable from *P. cactorum* by its predominatingly non-papillate sporangia', though he followed Tucker in deciding that *P. citricola* (culture from Baarn) was undoubtedly *P. cactorum*. Ashby, in unpublished notes kept at the Commonwealth Mycological Institute, wrote: 'The variety *applanata* has non-papillate sporangia. Its high temperature relations distinguish it from *P. syringae*. *P. citricola* seems to be a strain of this variety'.

Since its first description *P. citricola* has been recorded only three times, as far as I have been able to determine from the literature, once on *Malus pumila* var. *domestica* in Formosa (Sawada, 1931), once on *Citrus* in the Antilles (Cook, 1939), and once on grapefruit in South Africa, isolated by Doidge (Wager, 1940). Sawada (1927) also reported that the fungus caused infection when inoculated into *Euphorbia resinifera*, *Phaseolus vulgaris* and *Cydonia oblonga*. *P. cactorum* var. *applanata* has been reported on raspberry and *Primula japonica* (Herb. I.M.I. No. 32032) in Scotland (Dennis & Foister, 1942), on *Brachychiton populneum* and *Schinus molle* in Argentina (Frezzi, 1950), on *Hibiscus rosa-sinensis* and *H. schizopetalus* in Louisiana (James & Plakidas, 1951, 1952), on lemon (Smith, 1953), and on apple in England (by inoculation, Smith, 1953) and Holland (by inoculation, Buddenhagen, 1955). At C.M.I. there is a specimen (I.M.I. No. 39837), from Victoria, Australia, on *Ceanothus* sent by McLennan, and cultures isolated from antirrhinum in England (I.M.I. 51063) and New Zealand (I.M.I. 49559) and from gooseberry (I.M.I. 51062) in New Zealand by Smith (unpublished). The fungus causing blackroot of hops in New Zealand (Brien, 1938) and England (Keyworth, 1943) is undoubtedly the same as the foregoing. I have studied cultures of both these fungi. That of *P. citricola* (I.M.I. 21173) was in the National Type Culture Collection (N.C.T.C. 3057) taken over by the Commonwealth Mycological Institute in 1950, and it seems likely that it is a descendant of Sawada's original isolate. Its salient features are depicted in Pl. 12, figs. 3–5, 8–10, and they conform to the original description and figures. The cultures of *P. cactorum* var. *applanata* examined were I.M.I. 36353 (Pl. 12, figs. 6, 7), obtained from Baarn and listed as being from Chester's original isolate, and I.M.I. 24398, Frezzi's isolate (No. 789) from *Schinus molle*, submitted by Frezzi to Chester who confirmed the identification (Frezzi, 1950). Again, both conform to Chester's conception of the variety. I can detect no salient morphological differences between the three isolates, and I consider them to belong to the same species, *P. citricola* Sawada taking precedence.

It remains to be considered whether the morphology of *P. citricola* (syn. *P. cactorum* var. *applanata* Chester) is essentially that of *P. cactorum*, as suggested by Tucker and Leonian, or whether it is sufficiently distinct to

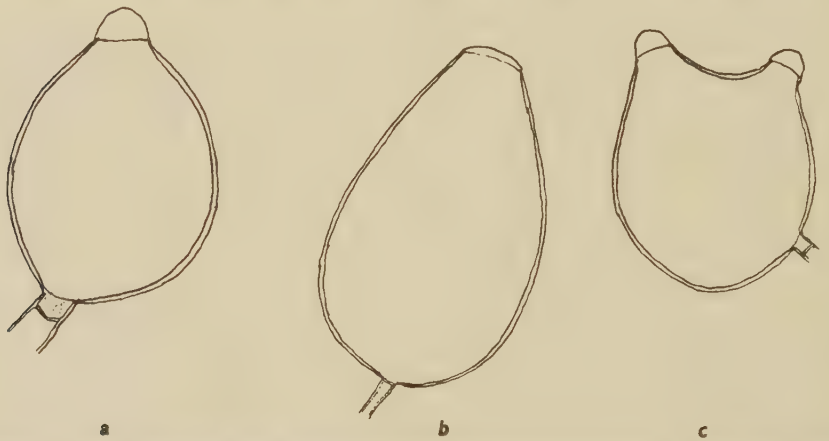
allow either the retention of a variety within *P. cactorum* or the resuscitation of Sawada's species. For this purpose the three isolates were compared with *P. cactorum* (I.M.I. 21168), the strain described by Blackwell (1934) as typical of the species.

The most characteristic feature of *P. citricola* is the non-protruding sporangial apex with shallow thickening. This has been noticed by all the workers quoted above and by others; it is a constant feature. The operation of killing and staining the sporangium causes the apex to flatten or even sink in. The hemispherical papilla and deep apical thickening characteristic of *P. cactorum*, which do not contract on fixing, are not seen on normally mature sporangia. Waterhouse & Blackwell (1954) have pointed out that the 'apical thickening is a useful diagnostic character for, at maturity, it is of constant shape however variable the size and shape of the sporangium'. This constant morphological difference should, therefore, preclude the assimilation of *P. citricola* into *P. cactorum*.

There are other distinctions between the two fungi which support this view. A number have been summarized for *P. c.* var. *applanata* by Frezzi (1950), who recognized the non-prominent apex and the non-pedicellate, non-caducous nature of the sporangia. He described the sporangial stalks as long, in contrast to the short ones of *P. cactorum*, and having swellings; the oogonia less frequently had an enlarged base, normally so in *P. cactorum*; chlamydospores were rarely or never produced, and zoosporangia were rare on solid media, whereas both are usually common in cultures of *P. cactorum* under these conditions. Of these characters, the non-caducous, non-pedicellate nature of the sporangium is a good diagnostic feature. The sporangiophores of *P. citricola* are commonly long and lax even on a solid medium, and they branch rather irregularly (Pl. 12, figs. 3, 9). There are swellings here and there, particularly at the points of branching. The sporangial stalks also are more often long than short, particularly in water; they are very thin and may have an occasional swelling (Pl. 12, figs. 4, 9). The cross-septum delimiting the sporangium is usually flush with the base and is without a septal plug; branching does not always occur immediately below it. In contrast, the sporangiophore system of *P. cactorum* is a neat and compact, fairly regular monochasial sympodium (Pl. 12, fig. 2), usually without branch swellings, though occasionally the point of branching may be very slightly enlarged. In moist air the sporangial stalks are very short and rather stouter than in *P. citricola*; in an aqueous medium they are longer, but always very much shorter than those of *P. citricola* when grown under the same conditions. The cross-septum delimiting the sporangium in *P. cactorum* is laid down in the stalk at a distance of 1–2 μ from the base of the sporangium, the space between the two being plugged with a transparent material chemically different from the septum (Text-fig. 3*a*). Branching occurs just below this septum. When the sporangium is shed the break occurs immediately proximal to the septum (Pl. 12, fig. 1). Shedding is frequent when the sporangia develop in moist air but less frequent in water. The sporangia of *P. citricola* are not naturally shed. If they become detached as a result of rough treatment, a longish piece of hypha accompanies the sporangium (Pl. 12, figs. 4, 5). When the two species

are grown side by side from comparable inoculum, the sporangia of *P. citricola* are larger; on hemp seed in Petri solution they reach up to 78 μ in length and 47 μ in width. It is noteworthy that Tucker, working with fifteen strains of *P. cactorum*, found (from measurements of up to 400 sporangia) that they never exceeded 55 μ in length or 39 μ in breadth; indeed, only one strain exceeded 35 μ in breadth (39 μ was the limit) and only four reached or exceeded 50 μ in length (51, 51, 55, 50 μ), and one of these at least appears to be typical *P. citricola*. Frezzi's measurements for sporangia were 21–69 × 16–46 μ.

The general shape is obpyriform, often elongated obpyriform, never spherical or ellipsoid, and is frequently distorted. The sporangia are sometimes divided from the apex by a fissure reaching nearly to the middle, or there appear to be two diverging apical growing points, each half having the characteristic apex (Text-fig. 2 (24); Pl. 12, fig. 6). These features are



Text-fig. 3. *a*, *P. cactorum*, outline of sporangium showing basal septum; *b*, *P. citricola*, outline of sporangium showing basal septum; *c*, *P. cactorum*, sporangium with two papillae. × 1000.

in direct contrast to those of *P. cactorum*, with its shapely spherical to ellipsoid (excluding the papilla) or obpyriform sporangia, that are very rarely distorted; though very occasionally they bear two papillae separated by a shallow dip (Text-fig. 3*c*) there are never two widely diverging apices as in *P. citricola*.

There are other points of difference in the vegetative phase, not perhaps of acceptable diagnostic value when considered alone, but when taken in conjunction with the more important features described above they add weight to the bias in favour of keeping *P. citricola* as a species. These are the more rapid growth of the mycelium (one and a half times to twice as rapid as for *P. cactorum*), particularly after the first 24 hr.; the slightly higher minimum temperature for growth (over 3° C. in comparison with under 2·5° C. for *P. cactorum*); the more radiate growth of the colony on agar which often has the appearance of a flower with pointed petals, in contrast to the uniformly fluffy appearance of *P. cactorum* colonies; and the very rare production of sporangia and chlamydospores on solid media.

The oogonia and oospores of these two fungi are remarkably alike, and this is what led Chester, Tucker, and others to consider them conspecific. The difference pointed out by Frezzi, viz. the less frequently enlarged base of the oogonium of *P. citricola*, does not seem to hold good on all media. The oogonial range given by Sawada for *P. citricola* was 21–44 μ, by Tucker 20–36·7 μ, by Chester 22–30 μ, and by Frezzi 17–38·5 μ; for the oospores the corresponding figures were 18–38 μ, 17·5–30·1 μ, 20–27 μ, and 14·5–35·5 μ. The oogonium of *P. cactorum* is very rarely smaller than 19 μ in diameter; only two of Tucker's fifteen strains produced oogonia smaller than this (18·4 and 17·5 μ) on a variety of media. The diameter is almost always over 20 μ; the maximum is rarely over 38 μ, only one of Tucker's strains exceeding this (38·5 μ); the commonest measurements lie between 27 and 29·5 μ. The only difference here is that the oogonia of *P. citricola* may be over 40 μ, whereas the limit for *P. cactorum* appears to be about 38·5 μ. In both, the oogonial wall is 1–1·5 μ thick, the oospore nearly fills the oogonium, leaving a space about equal to the thickness of the oogonial wall between the two, and it is slightly eccentric. The oospore wall is 2–2·5 μ thick and there is one large oil globule in the protoplasm. The only difference I have been able to detect between the two species is the slight yellow colour of the oogonial wall of *P. citricola* in pure culture, in contrast to the invariably colourless one of *P. cactorum*. This coloration is considerably deepened when the former species is grown on malt agar, the difference between the two then being very noticeable.

The antheridium, on the other hand, provides a distinction which I consider important in maintaining the two as separate species. The antheridial branch of *P. cactorum* arises either from the oogonial hypha itself, immediately below the wall delimiting the oogonium, or from the same parent hypha as the oogonial branch at a short distance, not usually greater than about the width of the oogonium. Thus it is closely monoclinous and the antheridium is applied to the oogonium at a point close to the oogonial stalk. The antheridial branch of *P. citricola* is often in such a tangle of hyphae (Text-fig. 2, 30) that one cannot detect its point of origin (when it may well arise close at hand). But when it is possible to see it clearly, it can sometimes be traced back to a point remote from the oogonial hypha. In three of Sawada's figures (Text-fig. 1, 16–18) the antheridial branch is shown as coming from the opposite direction to the oogonial hypha. Similarly, two of Chester's figures (Text-fig. 2, 27–28) show diclinous antheridia, as do nine of twenty oogonia drawn by Frezzi (1950). Of eighty-four oogonia examined by me, sixteen had diclinous antheridia (Pl. 12, fig. 7), three monoclinous, and the rest were in a tangle of hyphae making it impossible to detect the point of origin. The antheridium may be applied to the oogonium at some distance from the stalk as often as it is close to it.

Conclusions

The features described show that *P. citricola* can be readily distinguished from *P. cactorum* in its sporangial state by the nature of the apex, the size and shape of the sporangium, and the fact that it is not shed. The close similarity of the oogonia of the two species does not seem to be a sufficiently

good reason for considering them to be conspecific. Other species of *Phytophthora* with apparently identical oogonia but different sporangia are at present kept separate. For example, *P. syringae* has oogonia almost identical with those of *P. cactorum* but is legitimately distinguished by its different sporangia, as well as by other features. If the difference between the two fungi under consideration lay only in the sporangial morphology, a varietal distinction would be adequate to separate them, but as there are other characters by which they are distinguishable, such as the yellow colour of the oogonial wall, the diclinous origin of some antheridia, and cultural characters, it is considered that *P. citricola* should be retained as a species.

My thanks are due to Miss E. M. Blackwell for kindly reading the manuscript and for her helpful comments thereon; to Mr C. Booth for much assistance with the photography; and to members of the Staff of the Commonwealth Mycological Institute who have helped with the preparation of the paper.

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(*Facing p.* 357)

EXPLANATION OF PLATE 12

(All taken from living material.)

Fig. 1. *Phytophthora cactorum.* Sporangium shed with a very short pedicel, × 325.
Fig. 2. *P. cactorum.* Typical close sympodium of sporangia on hemp seed in Petri solution followed by distilled water, × 375.
Figs. 3, 4. *P. citricola.* 3, sporangia on corn meal agar, × 175; 4, single sporangium on the point of dehiscence, × 425.
Fig. 5. *P. citricola.* Sporangium on hemp seed in Petri solution followed by distilled water, × 600.
Fig. 6. *P. cactorum* var. *applanata.* Ditto. Sporangia with two apices, × 140.
Fig. 7. *P. cactorum* var. *applanata.* Oogonia with diclinous antheridia on corn meal agar, × 400.
Figs. 8, 9, 10. *P. citricola.* Sporangia on hemp seed in Petri solution followed by distilled water, × 400.

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BEHAVIOUR OF SOIL FUNGI IN THE PRESENCE OF FUNGAL ANTAGONISTS

By DAVID PARK

Department of Cryptogamic Botany, University of Manchester

(With 2 Text-figures)

Soil-inhabiting fungi are compared with exochthonous fungi in their reaction to fungal antagonism. In certain mixed cultures of fungi of both categories the soil-inhabiting fungi became dominant, and, after several days, demonstration of the presence of the exochthonous fungi became impracticable. Since the inactive spores of the exochthonous fungi were able to retain viability under conditions of more intense antagonism, their lack of success in these cultures is accounted for by the greater activity of the soil-inhabiting fungi. Under conditions which inhibited growth of soil inhabitants, added spores of exochthonous fungi remained demonstrable. It is suggested that the effect of environment on survival of at least some soil fungi is related to their tolerance for activity rather than to their total tolerance.

Introduction

The author (1955) has shown that fungi native to a particular soil differ from alien fungi in that they are able to maintain themselves and be saprophytically active in that soil, this difference being expressed under antagonistic conditions. In a comparison of the behaviour of these same fungi under conditions of bacterial antagonism (Park, 1957) differences were found between the vegetative activity of those fungi that were classed as soil inhabitants and of those classed as exochthonous fungi. However, fungi of both types were able to maintain themselves in the experimental cultures of that study.

The work described here enables a comparison to be made between the behaviour of soil inhabitants and that of exochthonous fungi under conditions of fungal antagonism.

Materials and methods

The twelve fungi used in this study are listed hereunder:

Natives:	*Mucor silvaticus*	Soil-inhabiting fungi
	Fusarium roseum	
	Monotospora daleae	
	Penicillium roqueforti	
	Cladosporium cladosporioides	
	Trichoderma viride	
Aliens:	*Rhizopus sexualis*	Exochthonous fungi
	Aspergillus niger	
	Botrytis cinerea	
	Penicillium digitatum	
	Trichothecium roseum	
	Stemphylium sarcinaeforme	Soil-inhabiting fungus

At the time the experiments were performed these organisms were classified as six native soil fungi and six alien fungi, the experiments being designed to compare aliens with natives. For this reason, each experiment comprised seven treatments; treatments 1 to 6 were inoculated with the individual alien species together with all six native species, while the inoculum for treatment 7 was all six aliens together with all six natives. The results are described in this form in the following pages. However, as in the author's 1957 paper, and for the reasons given there, the comparison that emerged was between soil inhabitants and exochthones. This contrast is made clear in the Discussion.

Agar, sand and soil cultures were set up as in the previous study (Park, 1957); the methods of observation were the same as those used there. Cultures were inoculated compositely with mixed spore suspensions of the organisms. A suspension of soil that had been exposed to propylene oxide vapour for 24 hr. and incubated 14 days was used as the source of bacterial antagonists.

Fungal Antagonists

On Czapek's agar

The developments observed in treatments which were inoculated with individual alien species are described first. *Rhizopus sexualis* sporangiospores germinated and the fungus was seen sporulating 20 hr. after inoculation; after 2 days, however, it could not be isolated. *Aspergillus niger* conidia failed to germinate, and groups of ungerminated spores could be seen up to the fourth day, but then became obscured by the dense growth of the native species; the fungus could not be isolated after 6 days. Conidia of *Botrytis cinerea* and of *Penicillium digitatum* remained dormant, and their presence could not be demonstrated after 5 days. *Stemphylium sarcinaeforme* conidia germinated, and gave rise to a sporulating mycelium which was maintained in the cultures. Germination of *Trichothecium roseum* conidia occurred and the fungus was seen sporulating up to the tenth day, after which its activity subsided; the fungus could not be isolated after 14 days.

The six native fungi grew and produced spores, and all were isolated at each attempt. After 14 days the mycelium produced had formed a deep, dense, opaque turf. Observations of all treatments were continued for 18 days, during which period the mycelium remained active, and did not show any great degree of lysis. On the eighteenth day the dishes, with the exception of the *Stemphylium sarcinaeforme* set, were re-inoculated with the appropriate alien species, and observations continued. No development of the added spores was seen, and after a further 8 days the presence of the five aliens again could not be demonstrated.

In sand substrata and in autoclaved soil

In both these substrata observations were made over a period of 16 weeks. The maximum determinable times of survival of the alien species are shown in Table 1, from which it will be seen that *S. sarcinaeforme*, like all the native species, survived throughout the experiment, while the remaining aliens were eliminated within 8 weeks.

Table 1. *Survival of alien species in the presence of all six native species**

Species	Sand + Czapek's soln.		Sand + soil soln.		Autoclaved soil	
	Aliens individually	All aliens together	Aliens individually	All aliens together	Aliens individually	All aliens together
Rhizopus sexualis	0	0	0	0	1	0
Aspergillus niger	6	5	7	7	7	6
Botrytis cinerea	2	3	2	2	2	2
Penicillium digitatum	4	3	3	3	5	4
Stemphylium sarcinaeforme	16	16	16	16	16	16
Trichothecium roseum	5	5	6	6	7	5

* Survival times are given in weeks.

Observations at the seventh day showed that lysis of the produced fungal structures was quite extensive. At subsequent observations mycelium was seen rather infrequently. Thus the conditions of intensive antagonism in soil and sand substrata found previously in the presence of bacteria (Park, 1957) can arise as a result of fungal interaction alone. The lysis was greater in the mixed fungal cultures than in single fungal cultures on the same substrata.

Fungal plus bacterial antagonists

The experiments in this section parallel those in the previous section, but organisms from propylene oxide-treated soil were present.

On Czapek's agar

Rhizopus sexualis sporangiospores underwent no development, and after 3 days the fungus could no longer be isolated from the cultures. *Aspergillus niger* conidia were not seen to germinate, and became lysed; after 10 days the fungus did not appear in platings made from the cultures. Conidia of *Botrytis cinerea* remained dormant, and none was seen after 4 days; after 5 days the fungus could not be isolated. *Penicillium digitatum* did not develop, and after 5 days the fungus could not be isolated. *Stemphylium sarcinaeforme* was seen sporulating on the third day after inoculation; its activity continued up to the twelfth day, when most of the mycelium in the cultures had become lysed. The newly produced spores of this species remained inhibited on the surface of the agar, and many were viable on subsequent plating. *Trichothecium roseum* underwent no development, its spores being subject to lysis; after 8 days this fungus could not be isolated.

The types of behaviour described above for each alien species were closely followed in the treatments inoculated with the mixed suspension of spores of all the alien fungi.

All six native species germinated, grew, and sporulated in all treatments, and then became subject to the antagonistic conditions. After the twelfth day the fungi in all the cultures were largely inactive; many of the spores lying in and on the agar, however, were viable. This state was maintained to the 18th day, when the treatments, with the exception of the *Stem-*

phylium sarcinaeforme set, were re-inoculated with the spores of their respective alien fungi. No germination of the added spores took place but, although some of these became lysed, others remained viable (except in *R. sexualis*); on plating, cultures of the aliens could be obtained for a further 10 days, when the experiment was discontinued. *R. sexualis* could not be isolated 3 days after re-inoculation.

It may be noted here that in the first experiment (on agar substratum without bacteria) the native fungi were active, and the re-inoculated aliens failed to survive. On the other hand, in the present experiment the antagonism was more intense so that the native species also were inhibited, yet alien colonies continued to develop in platings made from the re-inoculated cultures. This indicates that lack of survival of the alien species is not related directly to the antagonistic conditions, but to the general fungal activity in the cultures.

In sand substrata and in soil

The soil cultures were made in Petri dishes of soil which had been exposed to propylene oxide vapour and incubated for 14 days. The maximum determinable times of survival of the fungi as interpreted from smear slides and platings taken over a course of 16 weeks are shown in Table 2. Again the only alien to survive for longer than 8 weeks was *Stemphylium sarcinaeforme*. This fungus, like the native fungi, remained viable throughout the experiment.

Table 2. *Survival of alien species in the presence of all six native species in culture containing bacterial antagonists**

	Sand + Czapek's soln.		Sand + soil soln.		Autoclaved soil	
Species	Aliens individually	All aliens together	Aliens individually	All aliens together	Aliens individually	All aliens together
Rhizopus sexualis	0	0	0	0	0	0
Aspergillus niger	3	2	6	6	7	7
Botrytis cinerea	2	2	3	2	5	3
Penicillium digitatum	2	2	4	4	7	7
Stemphylium sarcinaeforme	16	16	16	16	16	16
Trichothecium roseum	2	1	3	2	4	3

* Survival times are given in weeks.

Discussion

Results published by Jefferys, Brian, Hemming & Lowe (1953) were interpreted by those authors as implying that production of antibiotics is an important factor in the ability of fungi to be saprophytically active in soil. However, other authors (Anwar, 1949; Butler, 1953; Garrett, 1956; Park, 1956) contribute to the view that a low sensitivity to antibiotic action is as important in this respect as the ability to produce antibiotics. The sensitivity to adverse factors is conveniently discussed in terms of tolerance. Burges (1949) has noted that micro-organisms in general are more tolerant of environmental extremes than are higher plants; Garrett (1955) attri-

butes the wide tolerance possessed by most micro-organisms partly to their capacity for prolonged retention of viability in an inactive condition. However, the results from the present study show that fungi having a sufficiently wide tolerance in the inactive state may not maintain themselves in mixed cultures; in fact, most of the exochthonous fungi had, like the soil inhabitants, spores which tolerated highly antagonistic conditions, yet, where the soil inhabitants were growing together with the exochthonous fungi, these latter were eliminated from the cultures. This was not due simply to the intensity of the antagonistic factors, but was correlated with the contemporary activity of the soil-inhabiting fungi, which were able to increase their population level relative to that of the exochthonous fungi,

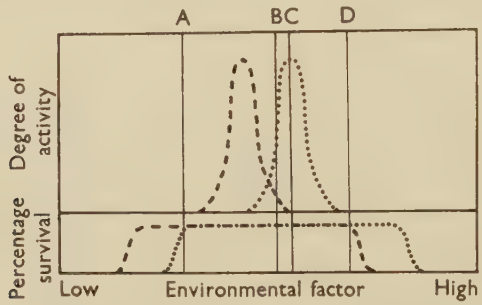

Fig. 1. Theoretical curves for survival and activity as affected by factors such as temperature: - - -, fungus I; . . ., fungus II.

until the presence of the latter could no longer be demonstrated. Stated simply, fungi able to retain a population of inactive spores, but not able to undergo active growth, were unsuccessful in these cultures. It thus appears that it is the limits of tolerance for vegetative and reproductive activity, rather than the limits of total tolerance, that are of primary importance in determining the range of environments in which an organism can maintain itself in nature.

This statement is best illustrated by reference to theoretical graphs. Fig. 1 shows curves representing percentage survival and degree of activity as affected by variation in a single environmental factor such as pH or temperature. It is understood (Hawker, 1950, p. 209) that the conditions allowing activity of a fungus are more critical than those allowing survival of its resting phase. For simplicity the curves are drawn symmetrical, the modes of the survival and activity curves for each fungus coinciding, and the shapes of the respective curves being identical for each fungus; the only difference is one of lateral displacement, i.e. position on the horizontal scale. Other factors are assumed to be constant and equally favourable for both fungi. It is clear that over the range A–D both fungi can survive by their resting stages. But at point C fungus II is active and may increase its population of spores, without any change occurring in the population of fungus I. Although theoretically fungus I will maintain its population of resting spores, in nature a considerable depletion of resting spore population must occur, thus increasing the relative preponderance of

fungus II over fungus I. Under continued persistence of these conditions, fungus I will come to comprise a negligible part of the population. At point B the conditions permit some activity on the part of fungus I but fungus II will still have a decided advantage and become dominant. At points A and D, where neither fungus is active, the dominance would be solely dependent on initial concentration of resting bodies, and the capacity of these to retain viability.

For factors such as tolerance to antibiosis, which has no effect at low and nil levels, the corresponding curves are of the type shown in Fig. 2. In the agar cultures plus bacteria described here, the condition obtaining from the 18th to the 28th day might be represented by line B. There the

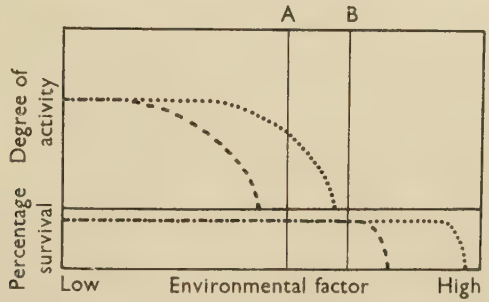

Fig. 2. Theoretical curves for survival and activity as affected by factors such as antibiosis: - - -, fungus I; . . ., fungus II.

degree of antagonism was such as to inhibit all the fungi present, yet added spores of exochthonous species survived and could be demonstrated by plating. In the corresponding cultures without the bacteria, however, the conditions were less antagonistic (line A, Fig. 2) so that the soil-inhabiting fungi grew and increased their population level, while the presence of the added exochthonous fungi could not be demonstrated.

In effect, it was found that the soil-inhabiting fungi were able to maintain themselves in the mixed cultures under conditions approximating to those of soil, while the exochthonous fungi were not. This is attributed to the lower 'tolerance for activity' possessed by the exochthonous fungi in respect of antagonistic factors.

The criterion adopted for the failure of a fungus to maintain itself in mixed cultures was the inability to demonstrate its presence by plating portions from the culture. This criterion indicated that the population of the fungus had dropped in relation to that of the other fungi present, so that its demonstration by standard methods was impracticable. However, there might still be, in such a case, some spores of the fungus present, i.e. although the fungus was recorded as having failed to maintain its population, it may not have been completely eliminated from the substratum. For the fungi used in this study it is unlikely that there exists any substratum markedly specific for the 'failed' organisms, and which, if added to the culture, would promote development of the fungi once more. Thus, for organisms of this type, the inability to isolate by standard methods indicates that the fungus no longer fulfils a significant role in the ecology

of the culture, so long as the environment remains reasonably stable. This would not necessarily be true for pathogenic fungi, for which the host plant acts as a specific substratum. This type of fungus, then, might remain pathogenically significant in soils, yet not be demonstrable by standard plating methods. Rishbeth (1955) cites such a case, and also points out that this could be due to the process of selection of suitable pathogenic strains from a heterokaryotic complex of different nuclear types. On the other hand, it could be due to straightforward survival of a small level of the pathogenic form, the presence of which is difficult to detect in the absence of the host plant.

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WORTMANNIN, AN ANTIBIOTIC PRODUCED BY *PENICILLIUM WORTMANNI*

By P. W. BRIAN, P. J. CURTIS, H. G. HEMMING and G. L. F. NORRIS

Imperial Chemical Industries Limited, Akers Research Laboratories, Welwyn, Herts.

(With Plate 13)

Wortmannin is an antibiotic isolated from a culture of *Penicillium wortmanni* Klöcker. It is not antibacterial. In its antifungal properties it is remarkably specific; of thirty-eight fungal species examined in spore germination tests, twenty-one were unaffected by 100 μg./ml., whereas five species (*Botrytis allii*, *B. cinerea*, *B. fabae*, *Cladosporium herbarum* and *Rhizopus stolonifer*) were inhibited by concentrations between 0·4 and 3·2 μg./ml. Wortmannin is a neutral substance, m.p. 240° C., very unstable in aqueous solutions of pH 3–8. It causes a characteristic spiral waving of germ-tubes of *B. allii*, easily distinguishable from that caused by griseofulvin.

Very few antibiotics have been obtained in pure form from species of the section Biverticillata-Symmetrica of the genus *Penicillium*. This paper describes an antibiotic obtained from a strain of *P. wortmanni* Klöcker.

Materials and methods

One strain only of *P. wortmanni* has been used: no. 989: received as *Penicillium* sp. in 1952 from the Rubber Research Institute of Malaya; a contaminant in a plate culture inhibiting the development of *Colletotrichum* sp.

Colonies of no. 989 on Czapek-Dox agar were rather restricted, thickly floccose, slightly raised and radially folded. The mycelium was mainly pinkish buff, with yellow outgrowths in places; the colony had a narrow white edge. A few penicilli could be found near the edge of the colony. The colony reverse was reddish orange or light-brown in colour. There were occasional colourless or light amber drops on the colony surface; there was little or no odour. Perithecia were absent. On malt agar the colonies were flat and more broadly spreading, yellow in colour, mainly owing to free development of bright yellow perithecia enmeshed in yellow mycelium. Penicilli were scarce. The colony reverse was deep yellow or brownish orange.

The parts of the penicilli were characteristic of the Biverticillata-Symmetrica in morphology, but the penicillus as a whole was frequently rather reduced in complexity, asymmetric or even mono-verticillate penicilli being not uncommon. The conidia were elliptical, smooth, 2·5–3 μ in long axis. The asci, of 6–8 μ diam., were borne in short chains;

the ascospores were elliptical, smooth or delicately spinulose, 3·5–4·0 μ in length. The perithecial initials, easily found on malt agar colonies, showed a structure typical of *P. wortmanni*. This culture was submitted to Prof. K. B. Raper, who agreed that it was *P. wortmanni*.

Methods of culture and assay of antibiotic activity have been previously described (Curtis, Hemming & Unwin, 1951).

Results

Production of antibiotically active culture filtrates on liquid media

The development of antifungal activity and the pH drift in cultures of *P. wortmanni* no. 989, on Czapek-Dox and Raulin-Thom media with various main carbon sources (Raulin-Thom also contains some tartrate which may be used as a carbon source), is illustrated in Table 1. It will

Table 1. *Development of antifungal activity and* pH *drift in cultures of* Penicillium wortmanni (*strain* 989) *on Czapek-Dox* (*CD*) *and Raulin-Thom* (*RT*) *media* (*initial* pH 5·5–6·0), *with various main carbon sources*

		Days' growth				
		6	10	17	20	31
Medium	Carbon source	BA units/ml.*				
CD	2·5 % glucose	—	16	32	16	16
CD	5 % glucose	—	16	32	64	32
CD	10 % glucose	—	8	16	8	8
RT	2·5 % glucose	—	32	8	8	16
RT	5 % glucose	—	32	64	64	32
RT	10 % glucose	—	32	64	128	64
RT	5 % sucrose	—	16	32	64	16
RT	5 % lactose	—	32	16	32	32
RT	5 % glycerol	—	16	32	64	16
		pH				
CD	2·5 % glucose	5·9	4·3	5·1	5·5	5·1
CD	5 % glucose	5·7	3·8	3·1	3·3	3·6
CD	10 % glucose	5·7	3·9	3·4	3·6	3·8
RT	2·5 % glucose	4·0	3·9	5·3	6·1	6·5
RT	5 % glucose	4·8	3·4	4·3	5·4	5·8
RT	10 % glucose	4·8	3·4	3·0	3·6	4·2
RT	5 % sucrose	4·8	3·4	3·4	4·0	4·9
RT	5 % lactose	5·4	3·3	3·6	3·6	5·5
RT	5 % glycerol	4·4	3·4	3·2	3·9	5·3

* As estimated by *Botrytis allii* spore germination test.

be seen that all the media tested, except Czapek-Dox with 10 % glucose and Raulin-Thom with 2·5 % glucose, gave similar results. Growth was as good on lactose as on glucose or sucrose, which is unusual; the growth on glycerol was very rapid and heavy, and the mycelium of a particularly brilliant colour. A number of other media has been tried, with various nitrogen sources and various organic supplements, but in none of these was the level of activity any higher than the maximum of 64–128 BA units/ml. recorded in Table 1. In the *B. allii* spore germination test it was frequently noted that the germ-tubes were spirally waved. This effect is described in more detail below.

Isolation of wortmannin

The mould was grown either in surface culture on Czapek-Dox (5 % glucose) for about 20 days, or for 15 days on Czapek-Dox (7·5 % glucose) in an 80 l. stirred fermenter. In both the antibiotic was readily extracted from culture filtrates with chloroform, benzene or carbon tetrachloride, but not with petroleum ether. The best method was to extract twice with $\frac{1}{5}$th vol. carbon tetrachloride; needle-shaped crystals separated on concentrating these extracts or on treating the residues with ethanol. On recrystallization from carbon tetrachloride, ethanol or benzene a colourless neutral solid, m.p. 240° C., was obtained. This turned yellow in sunlight; it contained no nitrogen, sulphur or halogen; it reduced Tollen's reagent. This antibiotic, which we propose to call wortmannin, was obtained in yields of about 40 mg./l. from surface cultures and of 100 mg./l. from stirred cultures.

Table 2. *Fungistatic activity of wortmannin: least concentration* (μg./ml.) *reducing germination to* 5 % *in Weindling medium* (pH 3·5)

	Expt. (*a*)	Expt. (*b*)	Expt. (*c*)
Acrostalagmus cinnabarinus Corda	—	100·0	100·0
Alternaria sp. (*tenuis* group)	—	100·0	—
Aspergillus clavatus Desm.	—	—	50·0
A. flavus Link ex Fr.	—	12·5	—
A. niger van Tiegh.	12·5	—	—
A. sydowi (Bain. & Sart.) Thom & Church	—	25·0	—
A. terreus Thom	—	—	50·0
Botrytis allii Munn	1·6	3·13	6·25
B. cinerea Pers.	—	1·6	6·25
B. fabae Sardiña	—	6·25	—
Cephalosporium acremonium Corda	—	100·0	—
Cladosporium herbarum (Pers.) Link ex Fr.	—	—	0·8
Fusarium bulbigenum Cke. & Mass.	—	100·0	—
F. graminearum Schwabe	100·0	—	—
Phoma sp.	—	—	25·0
Rhizopus stolonifer Ehrenb.	—	—	6·25
Stachybotrys atra Corda	—	—	50·0
Stemphylium sp.	—	50·0	—

The following species were unaffected by wortmannin at 100 μg./ml.: *Absidia glauca* Hagem, *A. orchidis* (Vuill.) Hagem, *Aspergillus nidulans* (Eidam.) Wint., *Byssochlamys fulva* Olliver & Smith, *Fusarium caeruleum* (Lib.) Sacc., *Gibberella fujikuroi* (Saw.) Wr., *Gliocladium roseum* (Link) Thom, *Helminthosporium sativum* Pamm., King & Bakke, *Mortierella isabellina* Oud., *Mucor erectus* Bain., *M. ramannianus* Möller, *Paecilomyces varioti* Bain., *Penicillium digitatum* Sacc., *P. expansum* Link, *P. gladioli* McCull. & Thom, *P. wortmanni* Klöcker, *Thamnidium elegans* Link, *Trichoderma viride* Pers. ex Fr. (2 strains), *Verticillium glaucum* Bonorden, *Zygorhynchus moelleri* Vuill.

Antifungal and antibacterial activity of wortmannin

The results of germination tests with spores of thirty-eight species of fungi are summarized in Table 2. Wortmannin is highly specific in its antifungal activity. Of the thirty-eight species tested, twenty-one were unaffected by 100 μg./ml.; on the other hand, germination of five species was reduced to 5 % or less by concentrations of 6·25 μg./ml. or less, viz. *Botrytis allii*, *B. cinerea*, *B. fabae*, *Cladosporium herbarum* and *Rhizopus stolonifer*. The susceptibility of *Cladosporium herbarum* to this antibiotic has been confirmed with eight strains, all being inhibited by concentrations between

0·4 and 3·2 μg./ml. The antifungal activity of wortmannin is unaffected by the pH of the medium in spore germination tests in the range pH 3–8. It is not appreciably antibacterial. Growth of all of several species of Gram-negative and Gram-positive bacteria tested was unaffected by concentrations of 200 μg./ml.

In concentrations of the order of 0·1–1·0 μg./ml., wortmannin induces a characteristic spiral 'waving' of *Botrytis allii* hyphae, an effect reminiscent of that caused by griseofulvin (Brian, 1949). However, the 'wavelength' is notably less than that observed with griseofulvin, and no excessive branching or hyphal distortion has been observed (Pl. 13).

Stability of aqueous solutions of wortmannin

Solutions of wortmannin (100 μg./ml.) were made in McIlvaine's buffer adjusted to various pH values in the range pH 3–8. Samples were taken at intervals, adjusted to pH 3·5 and assayed by the *B. allii* spore germination test. The antifungal activity of these solutions, expressed in BA units/ml., are presented in Table 3. It will be seen that the activity of the solution falls to about half its original value in one day in the range pH 3–7; at pH 8 the fall is more rapid.

Table 3. *Fungistatic activity (BA units/ml.) of solutions of wortmannin (100 μg./ml.) in McIlvaine's buffer, stored at* 25° C.

	pH					
Days	3	4	5	6	7	8
0	128	128	128	128	64	128
1	48	64	64	64	32	32
2	48	32	32	16	32	8
3	32	32	32	32	32	4
5	16	16	16	8	16	2
7	16	16	16	16	4	0
12	16	8	8	4	2	0

We are indebted to Mrs K. Balcombe, Mr P. C. Bamford, Mr B. W. Fitzpatrick, Mr J. H. Petty, Mr G. C. Pick, Mrs E. M. Sheppard, and Mr R. G. Weaver for technical assistance.

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EXPLANATION OF PLATE 13

Germinating spores of *Botrytis allii* in Weindling's nutrient, pH 3·5, containing 0·2 μg./ml. wortmannin, showing characteristic spiral waving.

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(*Facing p.* 368)

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PULVILLORIC ACID, AN ANTIBIOTIC OBTAINED FROM CULTURES OF *PENICILLIUM PULVILLORUM*

By P. W. BRIAN, P. J. CURTIS, H. G. HEMMING and G. L. F. NORRIS

Imperial Chemical Industries Ltd., Akers Research Laboratories, Welwyn, Herts.

Pulvilloric acid is an antibiotic isolated from cultures of *Penicillium pulvillorum* Turfitt. Media containing nitrate, ammonium salts or casein hydrolysate as nitrogen source, and glucose, sucrose or glycerol as carbon source, were found suitable for production of pulvilloric acid; the best medium tried was Raulin-Thom with 5–10% glycerol as carbon source. Pulvilloric acid is extracted from acidified culture filtrates in yields of about 600 mg./l., and is non-specific in its antifungal activity; thirteen species of fungi were used as test organisms and spore germination was reduced to 5% or less in each, in the range 3·125–25·0 μg./ml. at pH 3·5. Its activity is dependent on pH, germination of *Botrytis allii* being inhibited by 1·6 μg./ml. at pH 2·4 whereas 50 μg./ml. was required to produce the same effect at pH 4·0. It is only slightly bacteriostatic at pH 7·0. Aqueous solutions are stable at 25° C. in the pH range 3·0–8·0.

We have recently shown (Brian, Curtis & Hemming, 1955) that *Penicillium raistrickii* Smith produces the antibiotic griseofulvin. We have therefore thought it desirable to examine other species in the *P. raistrickii* series for antibiotic production, and this paper gives an account of work with *P. pulvillorum* Turfitt. One strain of the species has been tested previously and found inactive (Florey, Chain, Heatley, Jennings, Sanders, Abraham & Florey, 1949).

Materials and methods

Methods of surface culture and assay of antibiotic activity have been described previously (Curtis, Hemming & Unwin, 1951). All cultures were grown at 25° C. For antifungal assay, culture filtrates were adjusted to pH 3·5 if more than 0·5 unit above or below that value.

The following strains of *P. pulvillorum* have been used:

No. 504: from J. H. Warcup (his no. B. 42), isolated from Breckland soil (Warcup, 1951).

No. 1115: from Centraalbureau voor Schimmelcultures, Baarn, Holland, (C.B.S.), Turfitt strain.

No. 1116: from C.B.S., strain NRRL 2082 (see Raper & Thom, 1949).

No. 1122: from Commonwealth Mycological Institute, Kew, I.M.I. 40595, received at C.M.I. from G. Smith as NRRL 2082.

No. 1124: a substrain from 504, characterized by a deep ochre-yellow pigmentation of the colony reverse on Czapek-Dox agar.

No. 1125: a substrain from 504, with a bright pink colony reverse on Czapek-Dox agar.

No. 1126: a substrain from 504, almost completely sterile, with colony reverse on Czapek-Dox agar white with occasional pink patches.

No. 1131: a substrain from 1115 with more typically rough conidiophores.

All these strains produced conidiophores and conidia characteristic of *P. pulvillorum*, except no. 1115, which produced almost smooth conidiophores; occasional sectors produced rough conidiophores and no. 1131 was obtained from such a sector. All produced typical soft sclerotia. All sporulated well except no. 1124, which was almost white in colour and produced conidiophores sparsely.

Results

Production of antibiotically active culture filtrates on liquid media

In preliminary work, strain 504 was found to produce culture filtrates inhibiting spore germination of *Botrytis allii* in dilutions of 1:16 or 1:32 and preventing growth of *Bacillus subtilis* but not that of *Bacterium coli*. In the *Botrytis allii* spore germination assay, subinhibitory dilutions caused a marked stunting of germ-tubes, the abnormal straightness of affected germ-tubes being quite characteristic.

In subsequent work seven further strains of *Penicillium pulvillorum* were used, including the three substrains derived from no. 504. All, with one exception, produced culture filtrates with antifungal and antibacterial activity and, judging by the characteristic appearance of germ-tubes in the *Botrytis allii* assay, the same antibiotic was involved in each case. Strain 1126 produced almost inactive culture filtrates; sterile or lightly sporing strains of other species have been found less effective than sporing strains for antibiotic production, e.g. *Penicillium terlikowskii* for gliotoxin production (Brian, 1946) and *P. raistrickii* for griseofulvin production (Brian *et al.* 1955).

Strain 1124 was characteristic of the active strains. The development of antibiotic activity in cultures of this strain on Czapek-Dox and Raulin-Thom media is illustrated by Table 1, and on Raulin-Thom media containing glucose, sucrose, lactose or glycerol as main carbon source in Table 2. Considering antifungal activity first, it is clear that there is little to choose between Czapek-Dox and Raulin-Thom media. Experiments with other media, not reported in detail here, have confirmed that ammonium salts, nitrates and hydrolysed casein are suitable nitrogen sources for antibiotic production. Growth was poor on lactose, and it was an unsuitable carbon source for antibiotic production. Glucose and sucrose were satisfactory for growth; they were also suitable for antibiotic production; in each case the 5 % level was somewhat better than the 10 % level. Glycerol was the best carbon source for production of antibiotic activity, though growth was no better than on glucose or sucrose.

Material from crude culture filtrates was run on paper chromatograms with various solvent systems and the antibiotics detected on the paper by bioassays with *Bacillus subtilis* and *Botrytis allii*. Only one active substance was found by each bioassay. In each solvent system the same R_F values were obtained for active material by both bioassays, so both antibacterial and antifungal activity are presumably due to the same substance.

Table 1. *Development of antifungal and antibacterial activity and* pH *drift in cultures of* Penicillium pulvillorum (*strain* 1124) *on Czapek-Dox* (*CD*) *and Raulin-Thom* (*RT*) *media* (*initial* pH 5·7)

Medium	Glucose (%)	Days' growth: 6	10	17	24	31
		BA units/ml.*				
CD	5	—	8	32	32	64
	10	2	16	16	8	16
RT	5	—	16	32	32	32
	10	2	2	4	2	2
		Diameter of inhibition zone in *B. subtilis* assay (mm.)				
CD	5	—	15	15	15	16
	10	10	18	17·5	17	16
RT	5	—	12	13	13	12
	10	14	14	13·5	12	10
		pH				
CD	5	7·2	4·5	4·7	5·4	5·4
	10	4·5	4·1	4·1	3·9	4·0
RT	5	3·9	4·3	6·4	7·3	7·2
	10	2·9	3·0	2·9	3·1	3·6

* As estimated by *Botrytis allii* spore germination test.

Table 2. *Effect of carbon source on development of antifungal and antibacterial activity and on* pH *drift in cultures of* Penicillium pulvillorum (*strain* 1124) *on Raulin-Thom medium* (*initial* pH 5·3)

Carbon source and concentration	Days' growth: 6	10	17	25	51
	BA units/ml.				
Glucose 5%	2	4	16	32	32
Glucose 10%	—	2	2	16	16
Sucrose 5%	4	2	16	64	32
Sucrose 10%	2	4	2	2	—
Lactose 5%	—	—	—	4	16
Lactose 10%	—	—	—	8	16
Glycerol 5%	—	32	64	64	64
Glycerol 10%	—	16	64	64	64
	Diameter of inhibition zone in *B. subtilis* assay (mm.)				
Glucose 5%	—	11	13	14·5	15
Glucose 10%	—	10	16	16	15
Sucrose 5%	—	—	15	19	20
Sucrose 10%	—	12	15	14	14
Lactose 5%	—	—	—	—	12
Lactose 10%	—	—	—	—	17
Glycerol 5%	—	18	23	23	23
Glycerol 10%	—	14	27	25	25
	pH				
Glucose 5%	3·6	4·2	6·9	7·1	7·2
Glucose 10%	3·6	3·2	3·1	4·2	5·4
Sucrose 5%	3·3	2·5	5·0	6·6	6·8
Sucrose 10%	3·1	3·0	2·8	3·0	3·1
Lactose 5%	6·3	6·8	7·0	6·9	6·9
Lactose 10%	6·9	7·4	7·4	7·1	7·2
Glycerol 5%	7·3	6·4	6·1	6·9	7·2
Glycerol 10%	6·9	5·6	5·3	5·3	5·2

Isolation of an antibiotic

The mould was grown on Raulin-Thom medium (5 % glucose) for 13 days. Culture filtrates were acidified to pH 3·5 and extracted twice with $\frac{1}{10}$th vol. chloroform. On evaporating these extracts and crystallizing the residues from ethanol, buff-coloured needles were obtained in yields of 600 mg./l. This solid turns bright yellow on exposure to air, or on keeping *in vacuo* over phosphorus pentoxide in the light or dark. A solution of the yellow or the buff solid is nearly colourless in methyl, ethyl or butyl alcohol but bright yellow in acetone, ether or dioxane. Crystallization of the solid from solvents other than methanol or ethanol was not successful. These results are consistent with the view that the substance isolated is a loose addition compound with ethanol, formed during the isolation procedure by a reaction which is readily reversed to give a bright yellow substance.

The compound isolated is an acid; it liberates carbon dioxide from sodium bicarbonate solution, and is extracted from chloroform solution by buffers of pH 6·5 and above. It contains no nitrogen, sulphur or halogen; an alcoholic solution gives a blue colour with ferric chloride; it does not reduce Tollen's reagent. We propose to call this compound pulvilloric acid.

Antibiotic activity of pulvilloric acid

The minimum concentration of pulvilloric acid in Weindling medium at pH 3·5 required to reduce germination of spores of a number of fungi to

Table 3. *Fungistatic activity of pulvilloric acid: least concentration* (μg./ml.) *reducing germination to* 5 % *in Weindling medium*, pH 3·5

Absidia glauca Hagem	6·25	*Penicillium digitatum* Sacc.	6·25
Aspergillus niger van Tiegh.	25·0	*P. expansum* Link	6·25
Botrytis allii Munn	12·5	*P. gladioli* McCull. & Thom	6·25
Fusarium caeruleum (Lib.) Sacc.	3·125	*Stemphylium* sp.	6·25
F. graminearum Schwabe	6·25	*Thamnidium elegans* Link	3·125
Mucor hiemalis Wehmer	6·25	*Trichoderma viride* Fr.	12·5
Myrothecium verrucaria (Alb. & Schw.) Fr.	25·0		

5 % or less, is shown in Table 3. There is little difference in the susceptibility of the various moulds, i.e. pulvilloric acid is non-specific in its antifungal activity. Germ-tubes of *B. allii* in the presence of subinhibitory concentrations of pulvilloric acid have the unusually straight, stunted appearance recorded earlier for culture filtrates. Its antifungal activity is dependent on pH (Table 4) in a way that is characteristic of weak acids (see Grove, 1953). In view of its low antifungal activity near neutrality it is not surprising that pulvilloric acid is not highly antibacterial in broth at pH 7·0; under such conditions growth of some strains of *Bacillus subtilis* and *Staphylococcus aureus* is inhibited by 100 μg./ml., but other strains of these species and all Gram-negative species tested were unaffected by this concentration.

An explanation of occasional discrepancies between the antifungal and antibacterial activity of culture filtrates (Tables 1 and 2) can now be

offered. These discrepancies always took the form of unexpectedly high antibacterial titres in conditions where antifungal activity was low. In all cases where this occurred the pH of the culture filtrate was low (*c.* pH 3·0). The *Botrytis allii* assay was unaffected by pH of culture filtrate, since the pH of the latter was always adjusted to pH 3·5. Culture filtrates

Table 4. *Effect of* pH *on fungistatic activity of pulvilloric acid: least concentration* (μg./ml.) *reducing germination of* Botrytis allii *conidia to* 5 % *in McIlvaine buffers*

pH	Least conc.	pH	Least conc.
2·4	1·6	3·8	25·0
2·6	3·125	4·0	50·0
2·8	3·125	5·0	> 100·0
3·0	6·25	6·0	> 100·0
3·2	6·25	7·0	> 100·0
3·4	12·5	8·0	> 100·0
3·6	12·5		

for antibacterial assays were not adjusted in pH, so that where they were of low pH the diameter of the inhibition zone might be increased. This has been confirmed experimentally; a 400 μg./ml. solution of pulvilloric acid in buffer at pH 7·0 produces an inhibition zone of 12 mm. and at pH 3·0 of 16 mm.

The antibiotic activity of culture filtrates can be completely accounted for by the yields of pulvilloric acid obtained.

Stability of pulvilloric acid in aqueous solution

Pulvilloric acid is a relatively stable antibiotic unless exposed to air. Solutions were made in McIlvaine buffers, in stoppered bottles, in a pH range of 3·0–8·0, stored at 25° C. and assayed daily by the *B. allii* assay after readjustment to pH 3·5. Even after 14 days there was no appreciable loss in activity. Solutions autoclaved for 15 min. at 15 lb./in.^{2} lost no activity at pH 4·0, 5·0, or 6·0, and not more than half their activity at pH 3·0, 7·0 and 8·0.

We are indebted to Dr J. H. Warcup for giving us a culture; to Mr G. W. Elson for paper chromatographic analyses of culture filtrates; to Mrs K. Balcombe, Mr P. C. Bamford, Mr B. W. F. Fitzpatrick, Miss P. Hooper, Mr J. H. Petty, Mr G. C. Pick, Mrs E. M. Sheppard and Mr R. G. Weaver for technical assistance.

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STUDIES ON VESICULAR-ARBUSCULAR ENDOPHYTES

I. A STRAIN OF *PYTHIUM ULTIMUM* TROW. IN ROOTS OF *ALLIUM URSINUM* L. AND OTHER PLANTS

By LILIAN E. HAWKER, R. W. HARRISON, VALERIE O. NICHOLLS* and ANGELA M. HAM

Department of Botany, University of Bristol

(With Plate 14 and 4 Text-figures)

A phycomycetous endophyte, forming typical vesicular-arbuscular mycorrhizas with the roots of *Allium ursinum* L. and of some other plants, is described. Long, irregular, aseptate hyphae run longitudinally through the intercellular spaces of the cortex and produce both vesicles and intracellular pelotons and arbuscules. Entrance takes place through root hairs or by direct penetration of the piliferous layer.

The endophyte was isolated by various methods, the most satisfactory of which was a section-embedding technique. Isolates of *Pythium ultimum* were almost invariably obtained by this method.

The isolates were able to use a wide range of carbohydrates. Growth was poor with ammonium salts or nitrates as sole source of nitrogen but good with asparagine or peptone. Branching and coiling of the hyphae were increased by extracts of host roots and/or thiamin, but extracts of bulbs and roots of *Allium ursinum* reduced the rate of growth of the endophyte in pure culture.

Owing to slow and irregular germination of seeds, *A. ursinum* was not suitable for inoculation experiments, and so other species of *Allium* were used. Inoculation with the isolates of *Pythium ultimum*, under certain conditions, led to development of typical hyphae and vesicles within the root and, in older seedlings, to formation of the characteristic arbuscules.

The relation between host and parasite and the possibility that similar associations with other host plants may be due to unrelated fungi are discussed.

Introduction

Fungal endophytes of a phycomycetous type have been observed in the roots, and occasionally in other parts, of a large number of unrelated plants, including representatives of angiosperms, gymnosperms, pteridophytes and bryophytes. These endophytes produce aseptate or sparsely septate hyphae which grow largely in the intercellular spaces of the host but may also penetrate the cells and develop into coils or pelotons. Typical vesicles and haustorium-like, much-branched organs, known as arbuscules, are also produced. Both vesicles and arbuscules vary in size and form, not only in different plants, but often in the same root. These phycomycetous endophytes are known collectively as the vesicular-arbuscular mycorrhizal fungi, but it is by no means certain that the fungi concerned in different hosts are identical or even closely related.

* Mrs J. Sankey.

A number of unrelated fungi, including species of *Rhizoctonia*, *Fusarium* and other soil fungi, has been isolated by various workers from plants containing a typical vesicular-arbuscular endophyte. No conclusive proof that any of these is identical with the endophyte has so far been obtained. A consideration of the morphology of the endophytes in the host suggests that they are almost certainly phycomycetous and are unlikely to be identical with any species having a regularly septate mycelium.

Treub (1884) considered that the endophyte of sugar cane was a species of *Pythium*, since this fungus consistently grew out from pieces of infected root kept in a moist chamber. *Pythium* was also isolated from strawberry roots by O'Brien & McNaughton (1928) but was not considered by them to be the endophyte. Some forms of *Palaeomyces* occurring in the roots of the Rhynie fossils (Kidston & Lang, 1921) show a close resemblance to *Pythium*. It has been suggested that those vesicular-arbuscular endophytes occurring in the gametophytes of present-day species of *Lycopodium* may be species of *Pythium* (Bruchmann, 1885; Goebel, 1887; Treub, 1884), but the identity of the majority of the phycomycetous endophytes with *Pythium* has not been generally accepted.

Peyronel (1923) drew attention to the resemblance of the vesicles of certain vesicular-arbuscular endophytes to the chlamydospores of species of *Endogone* and later (1924, 1937) claimed to have traced an organic connexion between fruit-bodies of *E. vesiculifera* and roots of species of *Viola*, *Epilobium* and *Caltha*. He provisionally referred this type of endophyte to the genus *Rhizophagus* (first proposed by Dangeard (1900) for a fungus in poplar roots) and thought it probable that *Rhizophagus* and *Endogone* had been derived from a common ancestor. Butler (1939) supported Peyronel's hypothesis but was not satisfied with his proof of the organic connexion between the fructifications of *Endogone* and the mycorrhizal roots. Recently Mosse (1953, 1956) has put forward more convincing evidence for an organic connexion between fruit-bodies of an unidentified species of *Endogone* and mycorrhizal roots of strawberry. Mosse reports the development of similar endophytes in roots of strawberry and apple following inoculation with spores of her species of *Endogone*. Owing to failure to grow the species of *Endogone* in pure culture, this claim cannot be wholly convincing, but it is supported by considerable circumstantial evidence.

A study of the morphology, identity and physiology of the vesicular-arbuscular endophytes of *Allium ursinum* and certain other members of the Liliiflorae was begun in 1950. The problem has proved to be one of great difficulty and final proof of the identity of the fungus concerned was not obtained until the spring of 1956. Further studies are in progress to determine the effect of the endophyte on the nutrition and ecology of the host.

Morphology of the endophyte within the host tissue

In Allium ursinum

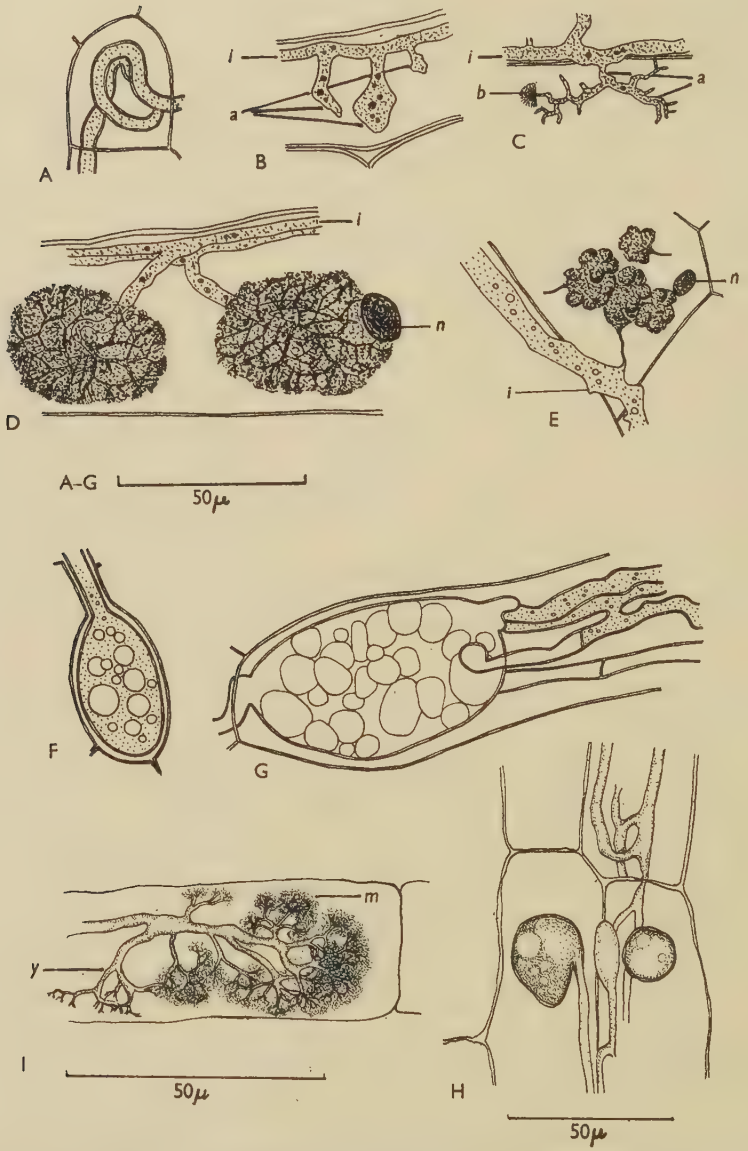
Roots of *A. ursinum* were collected at intervals throughout the year at Failand and Cleeve, Somerset, less frequently from various localities at Wotton-under-Edge, Glos. and Lyncombe Vale, near Bath, Somerset, and

occasionally from other localities in widely separated parts of England. Roots were usually, but not always, infected with the same type of fungal endophyte, and not all the roots of a particular infected plant contained the endophyte. There was no seasonal variation in the incidence of infected roots. The fungus does not penetrate bulbs, shoots or root apices, but is confined to the cortical tissues behind the apices of both contractile and fibrous roots. The vascular tissue is never invaded.

In fully established infections, the hyphae are found chiefly in the outer cortex to a depth of about 4–8 cells from the surface, but may extend for a considerable distance longitudinally. The main hyphae, which are aseptate (or with occasional septa in older hyphae), of irregular diameter (usually 3–6 μ), sparsely branched and filled with dense granular contents which stain deeply with aniline blue in lactic acid, are intercellular and tend to run longitudinally through the root for considerable distances in a manner reminiscent of *Peronospora*. These intercellular hyphae give rise to branches of two types which penetrate the cortical cells. Penetration into a cell is preceded by the development of a bulge in the host cell wall. Staining with resorcinol does not, however, indicate any change in the chemical constitution of the cell wall, of the type often produced as a result of the production of haustoria by species of *Peronospora* (Fraymouth, 1956). The penetrating hyphae within the cortical cells may be of two types (1) coiled 'pelotons' (Gallaud, 1905), or (2) finely divided haustorial-like branches or 'arbuscules' (Gallaud, 1905).

The pelotons (Text-fig. 1 A) vary in hyphal diameter and are often somewhat inflated, but the coils of hyphae are usually much looser and less well developed than those of the orchid endophytes. It is of interest that the hyphal type of haustorium produced in cells of the onion by *P. destructor* often shows a tendency to coil (Fraymouth, 1956; and personal communication). The pelotons of the endophyte of *Allium ursinum* may be of a similar nature.

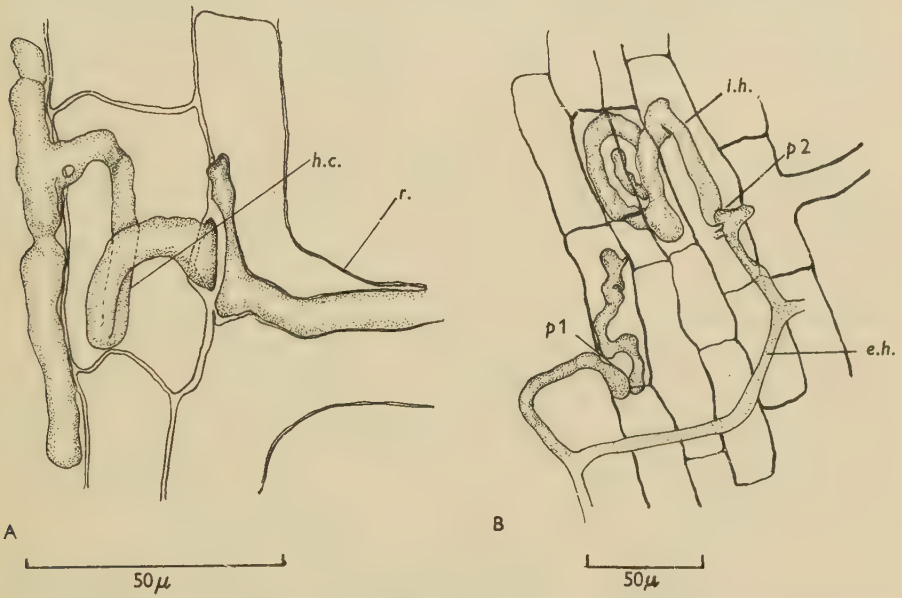
The second type of intracellular hypha, the characteristic arbuscule, is a much-branched structure. At the point of entry the arbuscular hypha may be slightly constricted, as with the haustoria of Peronosporaceae and some other parasitic fungi. The invading hypha first becomes inflated (Text-fig. 1 B) and later becomes repeatedly forked (Text-fig. 1 C). These early stages resemble the various types of haustorium produced by members of the Peronosporaceae (Fraymouth, 1956) but further branching, which may or may not be dichotomous, gives a much more complex structure than any seen in *Peronospora*. The ultimate ends of the branches may be so finely divided that they cannot be individually resolved (Text-fig. 1 D; Pl. 1, fig. 1). The invaded host cell may be completely filled with one or more arbuscules. The host nucleus may be enveloped but remains apparently unharmed. Finally the arbuscule becomes increasingly ill-defined and nebulous, through the coalescence and degeneration of the fine branch tips, until only a deeply staining mass, rich in oil drops, remains (Text-fig. 1 E; Pl. 1, fig. 2). Such an amorphous mass was termed a 'sporangiole' by Janse (1897), but this term is better reserved for the 'small sporangia without columellae, generally having a small number of spores' (Ainsworth & Bisby, 1954) characteristic of some members of the



Text-fig. 1. Morphology of the endophyte in the host. A–H, endophyte of *Allium ursinum*: A, peloton or hyphal coil; B, intercellular hypha, (*i*) with intracellular branches, (*a*) inflated prior to arbuscule formation. C, young arbuscule (*a*) showing coarse primary branching with fine branching beginning at (*b*). D, mature arbuscules, right-hand one in contact with host nucleus (*n*). E, disintegrating arbuscule (or 'sporangiole'). F, intercellular terminal vesicle, containing oil drops. G, intracellular, intercalary vesicle. A–G, del. V.O.N. × 500. H, younger vesicles in cortical cells. del. R.W.H. × 430. I, endophyte of *Endymion nonscriptus*, young (*y*) and fully developed (*m*) arbuscules in cortical cell of root. Tips of branches are already beginning to disintegrate. del. R.W.H. × 680.

Mucoraceae. Disintegration of the arbuscules continues until only a few irregular protoplasmic masses remain. When this stage is reached the intercellular hyphae are empty and septate, the points at which lateral branches arose being still visible as minute projections on the hyphae.

The vesicles, which have been described by many authors as characteristic of the phycomycetous endophytes, are less abundant in *Allium ursinum* than in some other host plants. They may be terminal or intercalary, and are occasionally cut off by a septum (Text-fig. 1 F–H). They



Text-fig. 2. Penetration of root of *Allium ursinum* by endophyte. A, L.S. root showing hypha in root hair (*r.*) and forming an intracellular hyphal coil (*h.c.*) in cortical cell. Note appressorium at base of root hair. del. R.W.H. × 680. B, surface view of part of piliferous layer, showing direct penetration at *p* 1 and *p* 2. External hypha (*e.h.*). Internal hypha (*i.h.*), del. R.W.H. × 300.

are usually thin-walled and vary in size (16–60 μ, usually 20–30 μ diam.) and shape, according to the configuration of the cell or intercellular space in which they are formed. The contents are dense, granular and rich in oil, but may become condensed to form a single deeply staining body. Smaller empty vesicles are sometimes formed and these may be detached from the parent hypha and lie freely in the host tissue. Vesicles are most abundant from August to January, during the period of maximum dormancy of the host.

Seedlings of *A. ursinum* usually remain uninfected for the first few weeks after germination but the endophyte is well established within the roots in 3-month-old plants. It is then possible to distinguish the numerous points of entry of the fungus. Entrance takes place either through a root hair (Text-fig. 2 A), or directly through the piliferous layer (Text-fig. 2 B). A slight swelling of the penetrating hypha usually occurs at the point of contact with the host root or root hair, and from this a thin penetration

hypha passes through the wall and, on gaining entry, enlarges to a diameter slightly greater than that of the external hyphae. Pelotons are formed rapidly in the outer three layers of cortical cells, but deeper penetration is almost entirely by intercellular growth. Pelotons are seldom found in the inner cortex and the formation of vesicles and arbuscules is usually delayed until the fungus has become well established. Penetration of roots of adult plants takes place in a similar manner at any time of year.

The general organization of the endophyte in the roots of *A. ursinum* is thus definitely phycomycetous. The irregular diameter of the intercellular hyphae and the morphology and development of vesicles and arbuscules suggest a closer affinity with the Peronosporales than with the Mucorales.

In other members of the Liliiflorae

The presence of an endophytic fungus in roots of species of *Allium* was reported by Gallaud (1905) and by Capelletti (1931). Samuel (1926) observed a similar fungus in a number of unspecified members of the Liliaceae. In the present investigation both wild and cultivated members of the Liliiflorae from a number of widely separated localities were examined. The endophyte was usually, but not invariably, present in roots of *A. ampeloprasum*, *A. japonicum*, *A. moly*, *A. pulchellum*, *A. schoenoprasum*, *A. ursinum*, *Asparagus officinalis*, *Convallaria majalis*, *Endymion nonscriptus*, (Text-fig. 1 I), *Eremurus bungei*, *Hemerocallis aurantiaca*, *Hosta* sp., *Lapageria rosea*, *Libertia ixiodes*, *Maianthemum bifolium*, *Paris quadrifolia*, *Polygonatum multiflorum*, *P. odoratum*, *Ruscus aculeatus*, *Scilla sibirica*, *Sisyrinchium* sp., and *Tofieldia palustris*. It was not found in *Allium rosenbachianum*, *Camassia esculenta*, *Iris pseudacorus* or *Muscari ramosum*.

The endophytes were essentially similar to that of *Allium ursinum*, but the vesicles varied in size according to the size of the cortical cells and intercellular spaces of the host roots, those in *Polygonatum multiflorum* reaching 160 μ in diameter.

In some other woodland plants

Woodland plants growing in the vicinity of the patch of *Allium ursinum* at Cleeve were examined. Hyphae together with arbuscules and/or vesicles were present in the roots of *Ajuga reptans*, *Geranium robertianum*, *Geum urbanum*, *Glechoma hederacea*, *Prunella vulgaris*, *Teucrium scorodonia*, *Veronica chamaedrys*, *Viola riviniana* and *Dryopteris filix-mas*. In other parts of the coombe these and the following additional species were mycorrhizal:* *Arum maculatum*, *Brachypodium sylvaticum*, *Buxus sempervirens*, *Epilobium roseum*, *Ilex aquifolium*, *Mercurialis perennis*, *Phyllitis scolopendrium*, *Prunus lusitanica*, *Senecio jacobaea*, *Sonchus asper*, *Stachys sylvatica*, *Taraxacum officinale* and *Viola reichenbachiana*. Godfrey (1956) described vesicular-arbuscular endophytes in roots of *Taxus baccata* and *Viola reichenbachiana* growing on limestone cliffs above the coombe in an area where fruit-bodies of species of *Endogone* were common. The endophytes of these plants, while generally similar, showed more variation in details of structure than did those of the Liliiflorae. Further work on some of them is planned.

* Information kindly supplied by Miss J. Darby and Mr M. Castle.

Isolation of endophyte

Isolation by plating-out pieces of surface-sterilized roots

Roots were washed in water, immersed in a solution of hypochlorite (¼ strength 'Milton') for 2–3 min., washed in sterile water and transferred to plates of plain agar. Subcultures were made from the tips of hyphae, which emerged after 2–3 days. By this method species of *Fusarium*, *Trichoderma*, *Rhizoctonia* and *Penicillium* were frequently isolated. It was unlikely that any of these septate fungi were identical with the endophyte. Strains of a species of *Pythium* were successfully isolated from *A. ursinum*, *A. moly*, *Convallaria majalis*, *Ruscus aculeatus*, *Scilla sibirica*, an unidentified species of *Hosta* and two cultivars of *Gladiolus*. Frequently, however, the *Pythium* was obscured by other fungi and bacteria, perhaps owing to its sensitivity to hypochlorite. Attempts to find a sterilizing agent which would favour *Pythium* rather than the contaminant fungi were unsuccessful. Bacterial contamination, however, was greatly reduced by addition of 0·2 % aureomycin to the culture plates.

Isolation from aseptically prepared longitudinal sections of infected roots

In order to be certain that any fungus isolated has actually grown out of the infected root and is not a chance surface contamination from the rhizosphere, a method whereby direct microscopic examination can be made is essential. Infected roots were held for 60 sec. in dilute 'Izal' solution (to avoid risk of injury to the fungus from hypochlorite) and thoroughly washed in sterile distilled water. Longitudinal sections were then cut under aseptic conditions and laid on a thin layer of 1·5 % water agar on a microscope slide, which was incubated in a damp Petri dish. Hyphae which grew out from the sections were then picked off.

This method was an improvement on the ordinary plating-out of pieces of root, but had the disadvantage that the method of surface sterilization of the roots was not entirely satisfactory. Contamination tended to occur while the sections were being examined. A further disadvantage was the rapid drying of the section.

The section-embedding technique

An improved technique was developed to overcome these difficulties (Harrison, 1955). No sterilizing agent was used, since the *Pythium* isolates are particularly sensitive to these. The roots were scrubbed with a test-tube brush under the tap and then transferred to a 250 ml. flask containing 100 ml. sterile water and a little sterile sand to act as an abrasive. The flask was then placed on a mechanical shaker for 10 min., after which the roots were transferred to a flask of sterile water without sand and again shaken for 10 min., followed by two further washings in sterile water. After this treatment the roots were free of external fungal hyphae and chlamydospores and were virtually sterile except for a few bacteria. Longitudinal sections were cut, or pieces of cortical tissue were teased out finely, under sterile conditions, and were placed on a sterile coverslip and covered with

a few drops of water agar containing 0·2 % aureomycin. The coverslip was then inverted on to a glass ring sealed to a slide. The sections were examined daily for growth of hyphae. When the endophytic hyphae could be clearly seen to have grown out into the surrounding agar medium, their tips were cut off and transferred to fresh agar medium. Isolates obtained by this method from *Allium ursinum*, *Endymion nonscriptus* and from the fern *Dryopteris filix-mas* were always a species of *Pythium*.

Isolations were most successful from young fibrous roots. The endophyte did not grow from contractile roots, although these were usually heavily infected. In general, only hyphae from fairly recent infections were still capable of extensive linear growth and where the arbuscular stage had been reached the fungus was apparently no longer active.

Morphology and identity of the isolates

Six isolates from *Allium ursinum*, two from *Gladiolus* cultivars and representative ones from *Allium moly*, *Convallaria majalis*, *Scilla sibirica*, *Endymion nonscriptus*, *Ruscus aculeatus*, *Hosta* sp. and *Dryopteris filix-mas* were grown on 3 % malt agar, on one or more of a number of other 'natural' media, and on 1·5 % water agar and glucose-peptone agar. Growth of these isolates, with the exception of one of those from *A. ursinum*, was essentially similar on any particular medium. Slight differences in vesicle size occurred between the various isolates, but these were of the same order as those between colonies of the same isolate on different media. The fourteen similar isolates closely resembled a stock culture of *Pythium ultimum* and their characteristics, when grown on 3 % malt agar, agreed with those given by Middleton (1943) for this species, with which they are, therefore, identified.

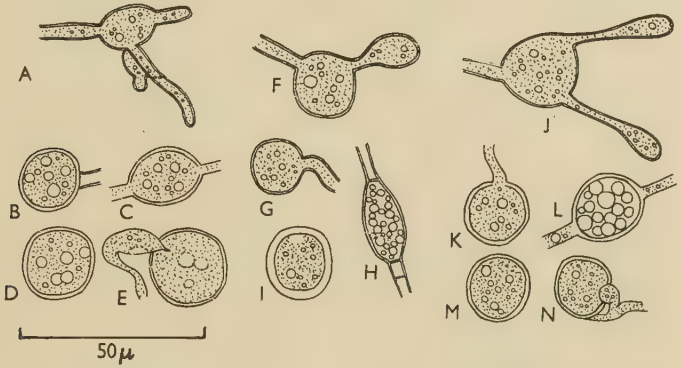
Colony characters. Abundant, white surface growth, filling a 9 cm. Petri dish in 4 days. After long periods in culture, growth becomes less uniform and aerial hyphae fewer. *Mycelium.* Hyphae 2–8 μ diameter, at first rarely septate, becoming septate in old cultures, much branched, branches arising at an angle of *c.* 45° with parent hyphae, constriction at base of branch absent or slight. *Sporangia.* Terminal or intercalary, thin-walled, spherical to pyriform (Text-fig. 3 B–D, G–I, K–M), contents granular or occasionally more or less organized into spherical bodies, sometimes, but not always, cut off from parent hypha by a septum, usually separated from parent hypha by disintegration of the latter, germinating by germ-tube (Text-fig. 3 A, F, J). Abundance, shape and size (10–28 μ diameter) varying in different isolates. *Oogonia.* Occasionally present in some isolates, terminal, wall thicker than that of sporangium, containing single spherical oospore which does not always fill oogonium, single antheridium occasionally present (Text-fig. 3 E, N).

One isolate from *Allium ursinum* differed from the others in its more irregular growth, with a tendency to irregular zonation and production of sectors, and in the production of small, thick-walled spherical spores of diameter 8–10 μ, resembling chlamydospores, in addition to the typical thin-walled sporangia. These differences are not considered to be sufficient to exclude this isolate from *P. ultimum*.

Physiology of endophytes

Rate of growth in culture

Linear growth rates of a typical isolate from *Allium ursinum* and of a stock culture of *Pythium ultimum* (isolated from damped-off lettuce seedlings) were compared on potato, malt, onion and lettuce extract agar media. The endophyte grew more slowly than did the stock culture. On a glucose-salts medium, both isolates grew slowly and there was little difference between them.



Text-fig. 3. Comparison of the endophyte in pure culture with *Pythium ultimum* isolated from damped-off lettuce seedlings. A–E, *P. ultimum*: A, hyphae growing out from vesicle, B, terminal and C, intercalary vesicles. D, vesicle (conidium) lying freely in medium. E, oogonium and antheridium. F–I, endophyte isolated from *Convallaria majalis*, as A–D. J–N, endophyte isolated from *Scilla sibirica*, as A–E. A–N, del. V.O.N. ×480.

Nutritional requirements

Carbon source. Glucose, fructose, sucrose, starch or inulin were utilized by all the isolates, but branching was most profuse on glucose or fructose media.

Nitrogen source. Nitrates or ammonium salts were poor sources of nitrogen. Asparagine or peptone were good. In this respect, the isolates resembled the stock culture of *P. ultimum* and a number of other cultures of *Pythium* and *Phytophthora*.

Mineral requirements. Magnesium sulphate and potassium phosphate (K_3PO_4) satisfied the mineral requirements of the endophyte in the presence of glucose and peptone. No attempt was made to free the medium from trace elements.

Factors influencing branching and coiling of hyphae

Cultures of the endophyte were examined for structures resembling the branched arbuscules and coiled pelotons produced within the host tissues. No such structures were seen in culture but branching and coiling of the hyphae were greater on some media than on others.

It is likely that the formation of pelotons and arbuscules within the host cell is due to the presence of growth-regulating substances. The effects of

an extract of roots of *Allium ursinum* and of small quantities of thiamin (vitamin B_1) were tested. These substances were added to 2 % agar and the media were spread over glass slides, which were inoculated at one end and incubated in moist Petri dishes. The rate of linear growth was measured and the amount of branching and coiling was estimated arbitrarily. Dilute root-extracts, which were prepared by expressing the sap from frozen roots and sterilizing it by passage through a bacterial filter, increased the amount of branching and coiling. Vitamin B_1 similarly increased branching but had a much greater effect than that of the root extract in causing coiling. The maximum effect occurred when both extract and vitamin were added (Table 1; Pl. 1, figs. 3–6).

Table 1. *The effects of glucose, root extract and vitamin B_1 on branching and coiling of an isolate from* Convallaria majalis

Medium	Branching	Coiling
2 % agar	+	None
2 % agar + 4 μg. vitamin B_1 per 100 ml.	++	+
2 % agar + 8 μg. vitamin B_1 per 100 ml.	++	++
2 % agar + 0·5 % glucose	++	None
2 % agar + 0·5 % glucose + 4 μg. vitamin B_1 per 100 ml.	++	+++
2 % agar + 0·5 % glucose + 8 μg. vitamin B_1 per 100 ml.	++	+++
2 % agar + extract of *A. ursinum* roots	++	+
2 % agar + root extract + 4 μg. vitamin B_1 per 100 ml.	+++	++
2 % agar + root extract + 8 μg. vitamin B_1 per 100 ml.	+++	+++

Antibiotic effects of host plant organs and of their extracts on Pythium *isolates in culture*

Gäumann & Jaag (1945) showed that pieces of living tuber of the orchid *Loroglossum hircinum* placed on agar plates inhibited the growth of the mycorrhizal fungus, *Rhizoctonia repens*. Dead pieces of tuber were readily invaded by the fungus.

During the experiments on effects of *Allium* root extracts on branching and coiling of the endophyte hyphae, these extracts occasionally reduced the growth rate of the hyphae. The effect of host plant extracts on rate of growth of the isolates was then examined in greater detail.

Whole bulbs and roots of *A. ursinum* were thoroughly washed in cold water, dipped in boiling water for 2 min. and then embedded in water agar in Petri dishes, which were then inoculated with the fungus. After 2 days incubation at 20° C., the rate of growth of the fungus in the presence of the bulbs was greatly reduced, but nevertheless on reaching the bulbs the hyphae readily penetrated them. No retarding effect of roots on fungal growth was seen. Penetration of healthy bulbs in the field was never seen and it was thought likely that in the above experiment the sterilizing treatment had destroyed some of the antibiotic substance originally present.

To avoid the possible destruction of antibiotic substances by heat treatment, extracts of bulbs and roots were prepared by a freezing method; 30 g. of freshly collected bulbs or roots were sliced and placed in boiling tubes surrounded with solid carbon dioxide in an insulated jar. The tissue was frozen for 1½ hr., and then thawed by holding the tubes under a

running tap. It was then wrapped in muslin and the sap was expressed in a mechanical press. The extracted juice was made up to an appropriate volume with distilled water, sterilized by Seitz filtration, added to 2 % agar cooled to a temperature just above the setting point and poured into Petri dishes. The quantities used were adjusted to give 1/80 and 1/40 dilutions of the Seitz filtrate for bulb and root extracts, respectively. Table 2 shows the effect of these extracts on two isolates of *Pythium ultimum* from *Allium ursinum*. The bulb extract retarded growth of both these. The root extract, at the strength used, was even more effective. Both extracts completely inhibited growth of isolates of *Pythium* obtained, by the hemp seed bait technique, from soil at Cleeve Coombe (i.e. from the neighbourhood of the patch of *Allium ursinum*).

Table 2. *The effect of extracts of bulbs and roots of* A. ursinum *on isolates of* Pythium *from the roots*

	Diameter of colony in cm.					
	Isolate I			Isolate II		
Days after inoculation	Control (no extract)	Bulb extract	Root extract	Control (no extract)	Bulb extract	Root extract
2	3·3	0·0	0·0	4·4	0·0	0·0
5	Plates full	1·3	0·0	Plates full	1·7	0·4
6	Plates full	2·0	0·0	Plates full	3·0	0·8
7	Plates full	2·5	0·0	Plates full	3·8	1·1
9	Plates full	3·5	0·0	Plates full	6·6	2·1
10	Plates full	4·2	0·0	Plates full	8·0	2·8
12	Plates full	6·1	0·0	Plates full	Plates full	4·2
14	Plates full	Plates full	0·0	Plates full	Plates full	Plates full

In other experiments with various concentrations of root and bulb extracts, some degree of inhibition of all isolates tested was obtained, but inhibition of the soil isolates was not uniformly greater than that of those from roots.

These experiments did not support the view that the failure of the fungus to colonize the bulb is due to a greater concentration of the inhibitory substance in the bulb than in the root. The substance might, however, be distributed unequally in the bulb. Local high concentrations in the outer layers and at the junction of bulb and roots might provide a barrier to penetration. The possibility of a difference in the mechanical resistance to penetration of cell walls in root and bulb must not be overlooked, in seeking an explanation of the immunity of bulbs to attack.

The nature of the inhibitory substance is not known. Attempts to isolate it and to identify it with an antibiotic found in garlic by Stoll & Seebeck (1951) were unsuccessful. An extract of the bulbs of *Endymion nonscriptus* had similar antifungal properties to those of the *Allium* extracts.

Inoculation experiments

Germination of seeds of *A. ursinum* took place only after a long period of dormancy. Simultaneous germination of a large number of seeds could not be obtained, even by the use of freezing and other methods designed to break the dormant condition, so that this species was not suitable for large-

scale inoculation experiments. Other species of *Allium* and *Scilla sibirica* were used instead.

Conditions permitting the establishment of a balanced mycorrhizal relationship between host and parasite are always critical. Relative vigour of the two organisms must profoundly influence the nature of the relationship and this in turn must be influenced by external factors. In inoculation experiments with the various isolates and with the stock culture of *Pythium ultimum*, the composition of the medium in which the seedlings were grown was varied.

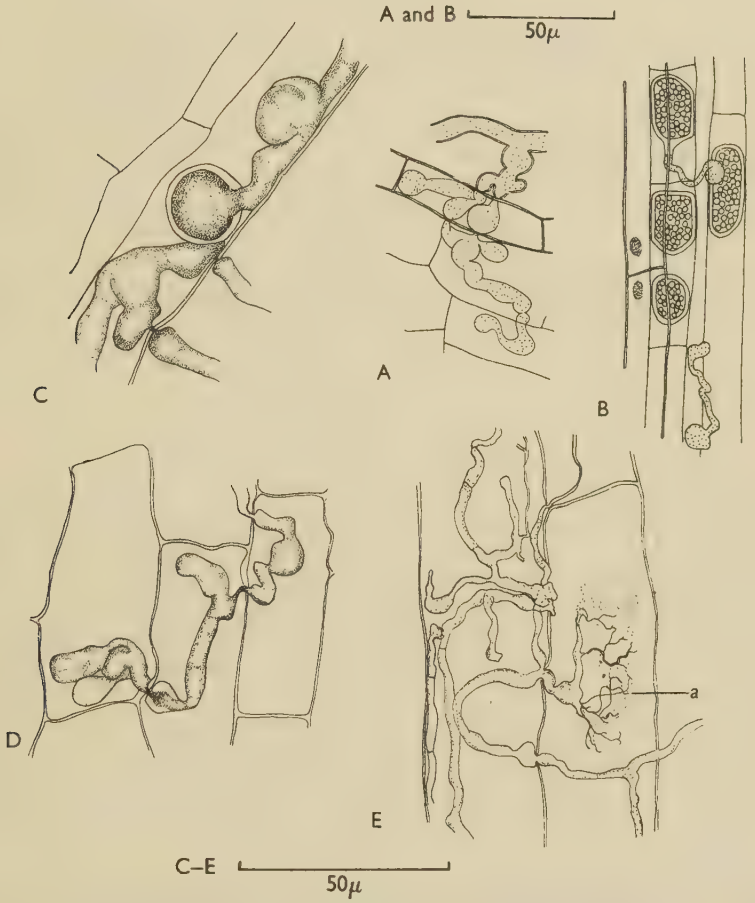
In preliminary experiments with *A. schoenoprasum*, *A. sativum* and *Scilla sibirica*, seeds were surface-sterilized with calcium hypochlorite by Knudson's (1925) method and placed in plugged tubes (five seeds per tube) containing sloped water agar (1·5 %), seedling agar ($Ca_3(PO_4)_2$, 2·0 g.; $NaNO_3$, 1·0 g.; KH_2PO_4, 0·5 g.; K_2HPO_4, 0·5 g.; $MgSO_4$, 0·29 g.; NaCl, 0·1 g.; $FeCl_3$, 0·01 g.; agar, 10 g.; distilled water, 1 l.), or McArdle's agar (glucose, 2·0 g.; maltose, 2·0 g.; soluble starch, 2·0 g.; $MgSO_4$, 0·5 g.; $Ca(NO_3)_2$, 0·5 g.; KH_2PO_4, 0·5 g.; agar, 15 g.; distilled water, 1 l.). When the seeds germinated, they were inoculated by placing small pieces of a water agar culture of a particular isolate on the surface of the agar slope. All the isolates tested, and the stock culture of *Pythium ultimum*, grew across the agar and reached the roots. Spherical appressoria were formed on the root surface and the fungi penetrated the first few layers of the root (Text-fig. 4 A). Vesicles were formed within the root at 7–14 days after inoculation (Text-fig. 4 B). After 3–4 weeks, the roots began to disintegrate and the seedlings were discarded. No arbuscules were formed during these experiments.

In an experiment with *Allium sativum*, grown on complete McArdle's agar and on the same medium lacking various constituents, and inoculated with the stock culture of *Pythium ultimum* and with the isolate from gladiolus roots, respectively, penetration was most rapid on the complete medium and did not take place, or was greatly delayed, on the medium lacking carbohydrate. This experiment indicated that penetration is most vigorous when the mycelium is well nourished, i.e. in the presence of carbohydrate and nitrate.

A series of inoculation experiments was also undertaken with *Allium cepa* on McArdle's agar and on this medium lacking various constituents. The results largely confirmed those of the preliminary experiments. Arbuscules did not develop in any of the inoculated seedlings but typical hyphae (Text-fig. 4 D) and vesicles were present. Good nutrition was essential for penetration.

The failure in these experiments to reproduce exactly the mycorrhizal condition, as seen in naturally infected roots, might have been due to a number of different causes. Assuming that the isolates of *Pythium* are identical with the endophyte, which is made highly probable by the method of isolation, then (i) *Allium cepa* might not be susceptible to attack or capable of forming the mycorrhizal association, (ii) the seedlings may have been too young so that the endophyte had not become fully established before the end of the experiment, or (iii) the conditions of inoculation may have been unsuitable.

Several species of *Allium* (see p. 380) form vesicular-arbuscular mycorrhizas similar to those of *A. ursinum*. Since fully developed arbuscules are not seen in naturally occurring mycorrhizal roots of young seedlings, it is likely that the mycorrhizal condition must be thoroughly established before



Text-fig. 4. Inoculation experiments. A, penetration through piliferous layer into cortex of root of seedling of *Allium schoenoprasum* by isolate from *Convallaria majalis*. B, later stage showing vesicles. A, B, del. V.O.N. ×380. C, endophyte in root of 4-week-old seedling of *Allium cepa* grown in soil taken from *A. ursinum* patch at Cleeve, Somerset. Vesicles present but no arbuscules formed. D, L.S. root of similar seedling grown in sterile soil inoculated with isolate from *A. ursinum*. C, D, del. R.W.H., ×560. E, arbuscule (*a*) formed in root of 8-week-old seedling of *A. cepa* grown in sterile soil inoculated with same isolate as in D, del. A.M.H. ×560.

arbuscules develop. Seeds of *A. cepa* were planted in soil taken from Cleeve Coombe, near infected plants of *A. ursinum*, and the seedlings were sampled at intervals. Hyphal penetration of the roots and the formation of vesicles took place (Text-fig. 4 C), but no arbuscules had formed in 6 weeks, i.e. in seedlings older than the oldest in the inoculation experiments.

Experiments of longer duration were therefore undertaken. Surface-sterilized seeds were sown in John Innes potting compost which had been steamed for 1½ hr. The pots also were steam-sterilized. The seedlings were watered with sterile water and kept in a heated glasshouse. After germination the seedlings were thinned to give twenty-five per pot, and 12·5 g. per pot of a sand plus 5 % oatmeal culture of *Pythium ultimum* (from *Allium ursinum*) was inserted into twenty boreholes made in the compost with a sterile glass rod. The seedlings were sampled at intervals. Infection took place freely, but arbuscules were not formed in any seedlings less than 8 weeks old. In 8-week seedlings grown under rather wet conditions, typical arbuscules developed (Text-fig. 4 E). Uninoculated seedlings grown under similar conditions were not infected. Thus, under suitable conditions after a sufficient period of time from inoculation, an isolate of *Pythium ultimum*, obtained from infected roots of *Allium ursinum*, was capable of causing the full development of a typical vesicular-arbuscular mycorrhiza. Further large-scale experiments on the effect of external conditions on the host-parasite relationship are in progress.

Discussion

Identity of the endophyte

The morphology of the endophyte in the roots of the Liliiflorae is strongly reminiscent of the downy mildews. The growth habit, irregular coenocytic hyphae running longitudinally through the root and putting haustorium-like processes into the cortical cells, is strikingly similar. This in itself suggests that the endophyte is likely to be a member of the Peronosporales.

The frequent isolation of *Pythium ultimum* by ordinary methods and, in particular, by the section-embedding technique, amounts to strong evidence that this fungus is the endophyte.

Furthermore, under certain conditions, inoculation of seedlings of *Allium* spp. with this fungus led to the development of a typical vesicular-arbuscular mycorrhiza, while control plants remained free from infection. Thus there is little doubt that strains of *Pythium ultimum* do form such mycorrhizas in roots of species of *Allium* and of some other members of the Liliiflorae.

Good evidence, however, has been put forward by Peyronel (1923, 1924, 1937) and Mosse (1956) in support of the identity of the vesicular-arbuscular endophyte of certain dicotyledonous plants with unidentified species of *Endogone* or related fungi. Complete proof is lacking, owing to the difficulty, and perhaps impossibility (Godfrey, 1956), of growing any species of *Endogone* in pure culture.

During the present study a number of dicotyledonous species, some ferns and a conifer, have been examined for the presence of endophytes. In many of these the endophyte is not exactly similar to that of *Allium* and in some it bears a striking resemblance to *Endogone*, in the form both of the mycelium and of the vesicles. One must conclude, therefore, either that both fungi are present in the same root and perhaps must *both* be present to give the typical appearance or, more probably, that the endophytic

fungus is not identical in all examples of vesicular-arbuscular mycorrhizas. Indeed it would be surprising if the same fungus occurred in so many different soils (woodland of various types, grassland, alpine regions, sand dunes, salt marshes, etc.) and was capable of penetrating and colonizing the roots of the numerous species known to show this type of invasion. The general similarity in appearance of the endophyte within the roots of different species might well be due to the formative influence of the restricted environment of the root tissues, and does not necessarily imply that the same fungus is always responsible.

Further work is in progress, or planned, with some of the species listed above (p. 380). Meanwhile it seems most likely that, while strains of *Pythium* are the normal endophytes of some members of the Liliiflorae, other fungi may play a similar part in other host plants.

The relation between host and parasite

It is of interest that, as with the better known examples of the ectotrophic mycorrhizas of trees and the endotrophic ones of the orchids, *P. ultimum* after penetration of the roots of *Allium* passes through an initial parasitic phase. After this, as in the other types of mycorrhiza, the host is able to control the invading fungus sufficiently to prevent further rapid penetration and sometimes to destroy it wholly or in part. The typical arbuscules do not develop until the active parasitic phase is over. As with the most complex haustoria among the Peronosporaceae (Fraymouth, 1956), the arbuscules are probably formed in response to host resistance. Certainly they finally disintegrate within the host cell (see p. 377). Moreover, at this stage, isolations of the fungus can seldom be made and the hyphae do not grow out of root sections, indicating that they are moribund. The present study suggests that the formation of antibiotic substances by the host provides a partial explanation for its control of the endophyte. Further work is being undertaken on this question. The conditions under which penetration leads to a prolonged period of parasitic activity and the death of the host, instead of to establishment of the mycorrhizal condition, are also being studied.

Other examples are known where a fungus which is an active parasite (or a near relative of such) of some plants forms a balanced mycorrhiza with others, e.g. *Rhizoctonia* spp. with many orchids and *Armillaria mellea* with *Gastrodia* and *Galeola*. The effect of environmental conditions on the nature of the relationship is obviously of great practical and theoretical interest.

There is no indication that this type of endophyte benefits the host directly. On the contrary, uninoculated seedlings grow as well as inoculated ones under certain conditions, although it is possible that conditions may exist under which the fungus is necessary for the adequate nutrition of the host. Possible indirect effects of the endophyte on the host are under investigation, and speculation at present would be premature.

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EXPLANATION OF PLATE 14

Fig. 1. Arbuscules in cortical cell of root of *Allium ursinum*. Note point of origin (*o*) from intercellular hypha. One arbuscule is in contact with host nucleus (*n*).
Fig. 2. Arbuscule disintegrating in cortical cell of root of *A. ursinum*.
Fig. 3. Hyphae of isolate from *Convallaria majalis* growing on 2% agar; note sparsely branched, straight hyphae.
Fig. 4. Same isolate on 2% agar+extract of roots of *Allium ursinum*. Note increased branching and slight tendency to coiling.
Fig. 5. Medium as in Fig. 4 but with addition of 8 μg. thiamin per 100 ml. Tendency to coil increased.
Fig. 6. On 2% agar+8 μg. thiamin per 100 ml. Coiling again pronounced.

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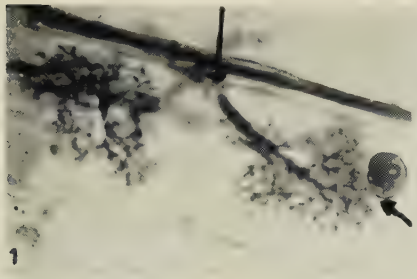
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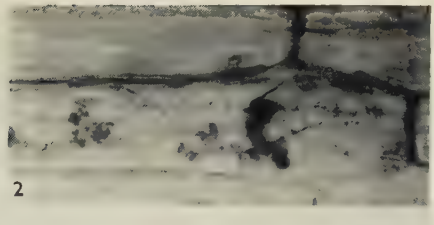
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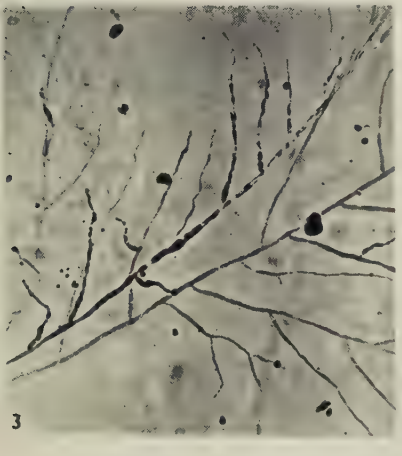
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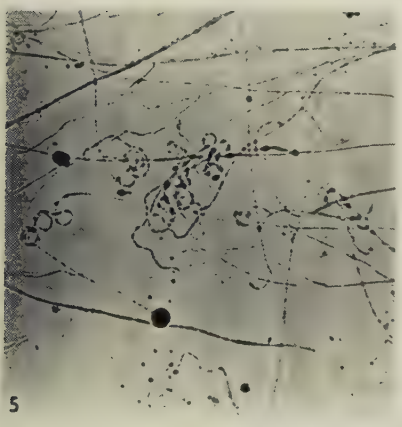
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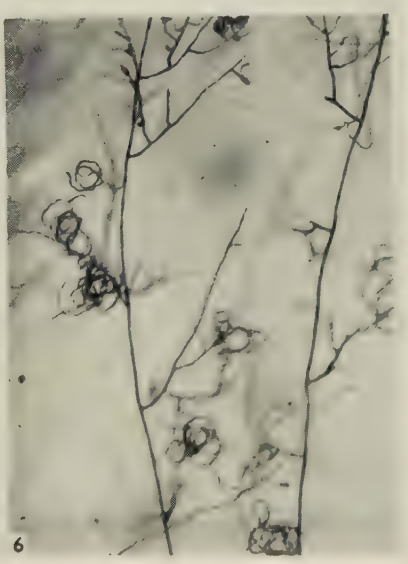
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(*Facing p.* 390)

Trans. Brit. mycol. Soc. **40** (3), 391–406 (1957).

DISTRIBUTION OF FUNGI WITHIN THE DECOMPOSING TISSUES OF RYEGRASS ROOTS

By J. S. WAID*

The Grassland Research Institute, Hurley, Berks

(With Plate 15 and 6 Text-figures)

Ryegrass roots, classified according to the degree of visible decomposition of the cortex, were washed in ten changes of sterile water and plated either intact, or after dissection, as fragments of outer cortex, or of inner cortex plus stelar cylinder. Mycelial populations in the three different root habitats were compared. The most active population originated from the root surfaces of intact roots. The progress of root degradation was paralleled by an increase in fungal activity in each zone, though the extent of this increase was not so great as the gradient of activity across the cortex. These results agreed with direct observations made on a semi-quantitative basis. A characteristic order of succession was found and the composition of the hyphal populations was different in each zone of the cortex. The primary colonizers were fungi with a low degree of competitive saprophytic ability; they included an endophyte, which was not isolated in culture. Secondary colonizers were fungi commonly isolated from soil habitats and having a high degree of competitive saprophytic ability.

Introduction

There is little doubt that the growth of grassland vegetation can bring about complex changes in the physical, chemical and biological status of agricultural soil. The decomposition of herbage plants results in the addition of a large quantity of organic material to the soil and the processes which occur during the breakdown of these materials are of interest to the soil scientist. Micro-organisms are one of the biological agencies concerned in decomposition and this investigation was planned to determine the distribution and ecology of the microflora of a decomposing plant structure.

Roots of ryegrass (*Lolium perenne*) were selected as suitable material because the bulk of the cortex can be easily dissected from the remainder of the root; thus the microfloras of the outer sheath (mainly cortex) and inner cylinder of tissue may be separately studied.

Materials

Roots of Aberystwyth S. 23 perennial ryegrass were obtained from 3- and 4-year-old ryegrass/white clover leys which had been cut at frequent intervals but had not been grazed by stock. The soil was a freely drained sandy clay loam of the Brown Forest group, having a pH value of *c.* 6·5 and a moderately low organic carbon content.

* Now at the Nature Conservancy, Merlewood Research Station, Grange-over-Sands, Lancashire.

Adventitious roots were cut away from the stem base and classified according to the degree of visible decomposition of the cortex. On this basis the following three types of root were recognized and sampled:

(1) Young white roots with intact apices and turgid cortical tissues.

(2) Light-brown coloured roots without lesions. These were relatively more abundant in winter months, when the environment of cold and waterlogged soils prohibits active decomposition of plant remains.

(3) Dark-brown roots with sloughing cortical tissues, generally bearing minor lesions; such roots were difficult to find.

The steles of light-brown roots with intact cortices probably function as conducting strands, since these roots have white healthy looking steles and very often have white apices. Decomposing roots without a cortex were not sampled.

Permanent preparations of longitudinal and transverse sections of roots were made in order to study the anatomical changes which accompany the breaking-down of the cortex. The firm cortex of the younger white roots consisted of living cells, but empty and apparently dead cells could be observed in the middle cortex of the older and decomposing roots. The cells in the middle cortex of light-brown roots had very thin walls, many of which were ruptured (Pl. 15, figs. 1, 2) so that there was open communication between adjacent cells upon the same radius. The inner cortex (2–3 layers of cells) and the outer cortex were composed of cells with thickened walls. The ease with which the outer cortex of ryegrass root can be stripped completely from the inner cylinder of tissues may be attributed to mechanical weakness in the middle cortex.

A determination of the proportion of outer cortex in dissected white and light-brown roots showed that it formed 56 and 35 %, respectively, of the total dry weight of the combined fragments. The reduction in proportion of cortical tissues in the older roots can be attributed partly to loss of the contents of cortical cells, and partly to the laying down of cell-wall material in the endodermis and stele.

The outer cortex of white roots turned a light-brown colour shortly after the roots had been dissected and exposed to air. Likewise, if roots were kept in water after dissection, the medium became tinged with brown. This suggests that autolysis may contribute to the changes in colour of semi-decomposed roots.

The only account of the changes in chemical composition of decomposing roots of ryegrass has been given by Baker (1955), who estimated quantities of soluble carbohydrates in the tissues of white and brown roots by the anthrone technique. Baker found that soluble sugars (glucose, fructose and sucrose) formed 6·10 and 1·56 % and fructosans 8·71 and 2·88 %, respectively, of the total dry weight of the two types of root.

The three populations of fungi which were sampled by cultural methods will be designated as follows. First, the *root surface* flora is that which developed from washed and intact roots. Secondly, the *outer cortex* flora is that which originated from the cortex sheath of dissected roots. And finally, the *inner cortex* flora is that which developed from the cylinder of inner cortex and stele of dissected roots.

Methods

Direct methods

Roots of ryegrass, carefully washed free of soil, were fixed and preserved in 'formal-acetic'. Selected roots were stained in an aqueous solution of cotton blue, mounted on slides, and their surfaces examined under the microscope. Records were made of the presence or absence (but not the density) of hyphae seen in 1 mm. segments of the root, beginning at the apex. The roots were then dissected and the cylinder of inner cortex and stele tissues was stained and examined in the same way.

Indirect methods

The methods used to wash roots were similar to those described by Harley & Waid (1955). Selected roots were placed in sterile water and freed of soil and debris by gentle agitation. They were transferred to fresh sterile water and this process was repeated until the roots appeared free of debris. The roots were then cut into 5 cm. lengths and, after all laterals had been removed, placed in phials of 30 ml. capacity with screwtops, together with 10 ml. sterile water. These phials were shaken at the rate of 300 beats per min. for ten periods of 2 min. each. Between each shaking period the water was either discarded, or retained for a determination of microbial content, and replaced with fresh sterile water.

When the fungal and bacterial content of the washing water was to be investigated, three separate 1 ml. aliquots were transferred from the samples by means of sterile pipettes to tubes containing 15 ml. molten agar at 40° C., agitated by hand and poured into Petri dishes.

In some samplings, washed roots were dissected in sterile water with the use of a sterile scalpel and forceps. The outer cortex and stele cylinder fragments were separated and plated, either immediately or after further washings.

The washed root material, whether whole roots or dissected fragments, was cut in sequence into 2 mm. segments, which were plated on nutrient agar. Five root segments were spaced apart upon each plate. The medium used was Czapek-Dox, pH 4·2, containing 5 g. yeast extract per l. (Warcup, 1950), and 10 g. glucose as the carbon source. The cultures were incubated at 25° C. for 3 days, and thereafter for a month at room temperature.

Recording of results. The following four methods of recording results were used:

(1) A semi-quantitative estimate was made, on the third day of incubation, of the growth on nutrient agar of the fungal mycelia from the root segments. If the colony diameter exceeded 1 cm. a score of 2 was given, if less than 1 cm. a score of 1 and if no colony was visible to the eye a score of 0. The total score (X) for any particular category of root was converted into '*an index of growth activity*', with a maximum of 200, calculated as follows:

$$\frac{X \times 100}{\text{no. segments in sample}}.$$

(2) The plates were examined at intervals during the following month. The number of records of each species was expressed as the number of times the fungus occurred on 100 segments of root; this is termed '*percentage occurrence*'.

(3) The sum total of all species of fungi was estimated by adding together all records, to give '*frequency per* 100 *segments sampled*', which indicates the size of the mycelial populations of the roots.

(4) The results of the microscopic and cultural methods of examination were compared by showing the presence of mycelia on segments as the '*percentage of segments inhabited by fungi*' regardless of the density or quality of the mycelia. This showed the proportion of root segments infected with fungi as estimated by either method.

Plates inoculated with root washings were examined after 3 days incubation, and the results of the fungal and bacterial counts were calculated as number of colonies per 10 ml. washings.

Results

Fungal and bacterial content of the root washings

Samples of the washings showed that the number of fungi in them decreased as the number of washing changes increased (Harley & Waid, 1955).

In a preliminary experiment five washing changes were found sufficient to cleanse the roots of the majority of detachable propagules, and relatively few micro-organisms appeared in further changes. The three categories of root were each equally cleansed by the washing method (Table 1); consequently ten changes of water were used in routine samplings. The washings contained many fungi which spore abundantly in culture and which were isolated frequently from the root surfaces, such as *Penicillium* spp., *Mucor hiemalis*, *Fusarium culmorum* and other Fusaria, *Mortierella* spp.,

Table 1. *Numbers of propagules detached from surfaces of ryegrass roots: sampled* 12 *May* 1954

Type of root	Numbers of fungi in duplicate samples	Number of washes	
		1	10
White (root apex)	A	423	14
	B	390	11
Light-brown	C	237	0
	D	143	0
Dark-brown	E	293	10
	F	160	5
	Numbers of bacteria		
White (root apex)	A	1458	28
	B	731	101
Light-brown	C	520	6
	D	182	7
Dark-brown	E	301	89
	F	253	4

Cladosporium herbarum, *Cephalosporium*/*Hyalopus* spp., and *Gliocladium roseum*. Species were also found, in the washings, which were not abundant in the flora of the root surface, e.g. *Papularia arundinis*, *Pullularia pullulans*, *Paecilomyces* spp., *Aspergillus* spp., *Verticillium* spp., *Oospora* spp., and *Heterosporium* spp. It may be assumed that these were components of microfloras associated with organic debris in the rhizosphere.

The composition of the fungal populations found in the washings varied from root to root but this was probably due to the mixed nature of populations from the root region (Harley, 1948).

Populations of root surfaces

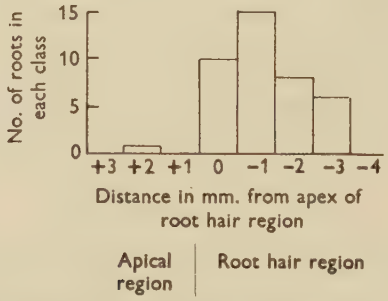
Microscopical examination of roots that had been washed in 10, 15, 20 or 40 changes of sterile water, respectively, revealed that most of the detritus originally associated with the root surfaces had been removed. Plant remains, together with their own floras of hyphae, spores and sclerotia, were sometimes seen entangled with root hairs. Mycelia were readily observed on the root surfaces; the population varied in density from root to root. Over the surface as a whole, the hyphae were septate and either hyaline or of a light-brown colour; small areas were often occupied by dense colonies of aseptate hyphae. Sclerotia and clamp-bearing hyphae occurred upon decomposing roots but no spores, spore-forming structures or rhizomorphs were seen. Bacterial colonies were present in clumps on the root surfaces, around root hairs and hyphae. Although a small proportion (25 %) of the root caps of white roots examined under the microscope was infected with hyphae, the surface of the first millimetre of root was always free of fungal hyphae. The fungi on the root cap may be assumed to be those which were active in the soil and had become attached to the root cap during its progress through the soil. The most distal hyphae occurring on the roots, excepting those seen on the root cap, were in the root hair zone; their distribution in relation to the position of the most distal root hairs indicates that root hair formation precedes fungal colonization of the root surface (Text-fig. 1 A).

Microscopical examination showed that the density of the hyphal population increased along the length of the roots (Text-fig. 1 B), and that the roots became 'fully colonized', i.e. at least one hypha per mm. root, at about 20 mm. from the apex.

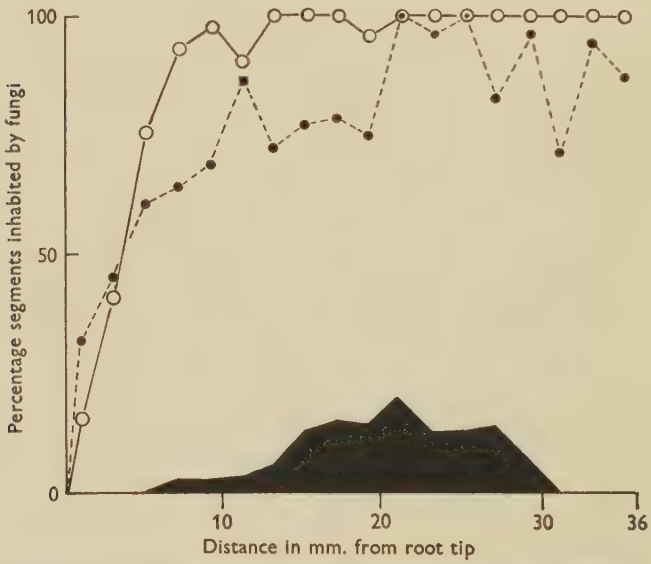
The general pattern of results obtained by the cultural (indirect) method confirms the direct observations. The results of the two sampling methods are compared in Text-fig. 1 B. The size of the surface populations estimated by the cultural method was lower than that observed by the direct method. This may have been due to variation in the material, or to errors arising from the indirect method. These errors may result from (1) treatment of material, e.g. vigorous washing and cutting may result in loss of hyphae; (2) failure of mycelium to grow on selective media, e.g. clamp-bearing mycelia were isolated from the inner cortex on two occasions only but were commonly seen within semi-decomposed cortical tissues; (3) competition between fungi in culture.

In Text-fig. 2, the density of all kinds of root surface fungi is plotted

together with the semi-quantitative estimate of mycelial growth of fungi isolated from corresponding segments of root. Both the activity and the total number of species of fungi increased with distance from the root tip.



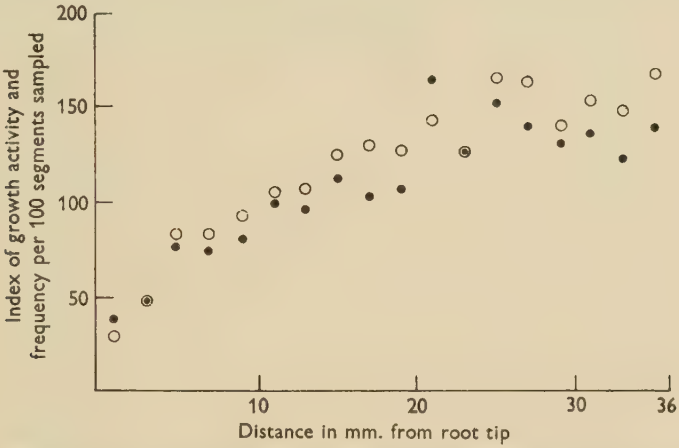
Text-fig. 1 A. Frequency distribution of the *most apical* fungal hyphae on the root surface, in relation to the segment containing the apical root hairs. Sample size 40 roots. Root cap infections ignored.



Text-fig. 1 B. A comparison of direct and indirect methods of sampling fungi on the apices of juvenile roots of ryegrass, and the distribution of a root endophyte in the inner cortex. −○−○−, Surface population observed by direct method; sample size 40 roots. - -●- -●- -, Surface population estimated by cultural method; sample size 75 roots. (Blocked in), Endophyte observed by direct method; sample size 40 roots. Population shown as the percentage of 2 mm. segments inhabited by fungi.

The distribution of Fusaria and Penicillia on the root apices (Text-fig. 3 A) was similar in pattern to that of the whole fungal population. In the proximal region of the root the Zygomycetes (Text-fig. 3 B) were relatively more abundant than the imperfect fungi, which was contrary to expectation since the former are held to be the first and dominant colonizers of fresh organic matter; a belief probably based on the fact that

Phycomycetes generally form *fruiting structures*, e.g. upon dung, before other groups of fungi, though Chesters (1949) seemed reasonably certain that some species of *Mortierella* occur on semi-decomposed root debris as a regular habitat.



Text-fig. 2. Estimates of the 'activity' and 'quantity' of the surface population of fungi on the apices of ryegrass roots, made by the cultural method. ○, Semi-quantitative estimate of the growth rate of mycelia, expressed as '*index of growth activity*'. ●, Total number of species of fungi, expressed as '*frequency per* 100 *segments sampled*'. Sample size 57 roots.

Flora of the dissected tissues of young roots

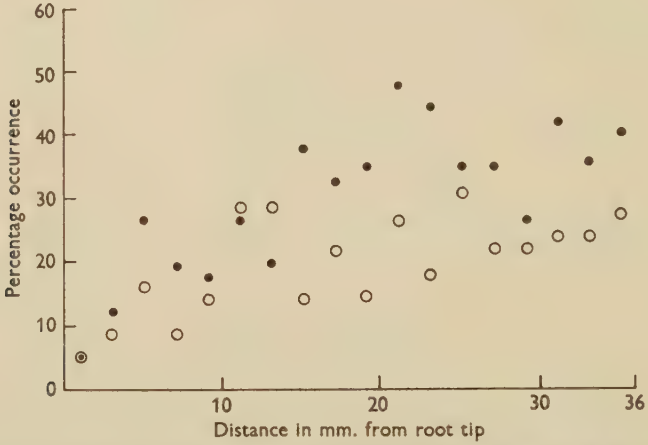
Most of the hyphae seen in the inner cortex were of one type; these hyphae were aseptate, thin walled, rarely branched and did not stain deeply (Pl. 1, fig. 3). Large groups of cortical cells were infected and the intracellular hyphae appeared to pass directly (without constriction) from one cell to another, but did not penetrate the endodermis. Infected cells were generally without nuclei and strands of granular material were often seen but no vesicles or other structures were observed within the roots. Vesicles borne on aseptate hyphae were seen on the root surfaces, but the hyphae were not seen to penetrate into the cortex. The endophyte has not with certainty been isolated nor seen to grow in culture.

Neill (1940) found a similar endophyte in the roots of field plants of *Lolium*; it was not the same as an intercellular and septate endophyte of the *aerial* parts. No endophyte was seen in the aerial parts of plants sampled at Hurley and examined by Neill's methods.

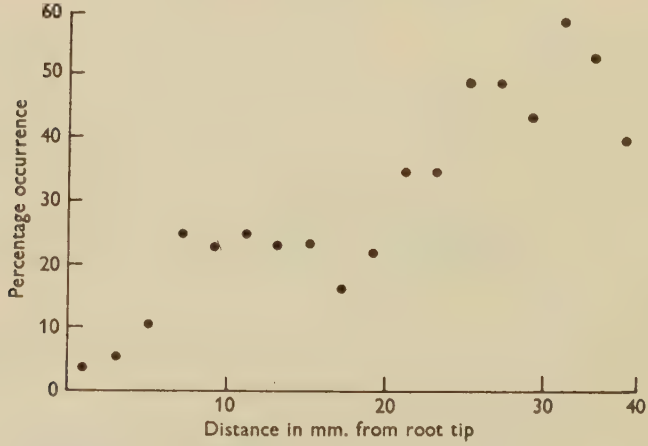
The description of the endophyte which has been given here agrees with that seen in the root cortex of wheat plants by Simmonds & Ledingham (1937) who observed it penetrating the root surface. Koch (1935), who saw the wheat endophyte, claimed that the fungus was similar to a Phycomycete, with arbuscules and vesicles, which occurs as an endophyte in the roots of various plants. The significance of these fungi has been reviewed by Harley (1950).

The endophyte was observed in the inner cortex and stele of only one-third of the roots examined (Text-fig. 1 B). It was generally less abundant

in short roots than in longer roots and the first 5 mm. of the dissected roots was free of infection altogether. It is of interest that the apical fragments of inner cortex and stele of ryegrass and cocksfoot (*Dactylis glomerata*), plated on nutrient agar, appeared free of fungal infection (Table 2), whereas the outer cortex was generally infected at a point far nearer the root tip.



Text-fig. 3A. The distribution of Fusaria and Penicillia upon the apices of ryegrass roots, sampled by the cultural method and shown as 'percentage occurrence'. Sample size 57 roots. ●, Fusaria; ○, Penicillia.



Text-fig. 3B. The distribution of Zygomycetes upon root apices, shown as percentage occurrence.

Microscopical examination also showed that the inner cortex tissue in the region nearest the stem was almost devoid of hyphae. The low incidence of hyphae in this region is possibly influenced by its proximity to the stem, which may enable it to become more resistant to fungal penetration through the relative abundance of metabolic materials in the stem.

Hildebrand & Koch (1936) plotted the percentage occurrence of the most distal and proximal infections of the endophytes of strawberry and tobacco. Maximum infections were observed from 7·5 to 10·0 mm. from the root tip and from 5·0 to 7·5 mm. from the root base in the case of strawberry, and from 2·5 to 5·0 mm. and from 0·0 to 0·5 mm. respectively in the case of tobacco. The root endophyte of ryegrass appeared to have a similar type of distribution as infection was only slight in the apical 10 mm. (Text-fig. 1 A) and proximal 5 mm. of roots (Table 3).

Table 2. *Number of consecutive apical segments (2 mm.) of dissected roots which appeared free of fungal infection after one month on nutrient agar. 5 October 1954*

	Ryegrass		Cocksfoot	
Root no.	Outer cortex	Inner cortex	Outer cortex	Inner cortex
1	1	3	2	5
2	2	3	1	7
3	0	8	0	10
4	0	5	0	3
Mean no. of segments	0·75	4·75	0·75	6·25
Equivalent length in mm.	1·5	9·5	1·5	12·5

Table 3. *Distribution of the most proximal hyphae of endophyte near root/stem junction, in white roots, October–November 1955. Inner cortex examined by 'direct' method*

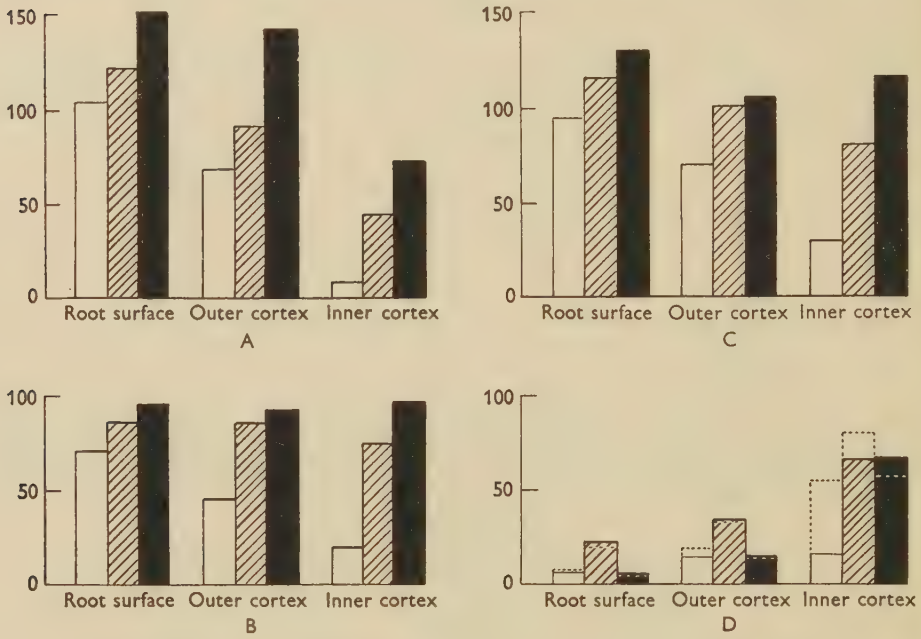
Distance of segment from base of root (mm.)	Frequency distribution of *most proximal* hyphae of endophyte in twenty-one infected roots	Number of infected 1 mm. segments out of sample of sixty roots
0–1	—	—
2	—	—
3	—	—
4	2	2
5	3	3
6	2	4
7	3	4
8	1	4
9	—	3
10	—	1
11	3	3
12	1	5
13	—	1
14	2	3
15	1	3
More than 15 mm.	3	

Sample size, 60 roots; number of roots infected in inner cortex, 21.

Floras of the three root zones

The majority of samples discussed in this section were made up of white root apices and light-brown roots. Dark-brown roots were only sampled in small numbers.

The results of the various observations made upon the fungal populations of the root surface, outer and inner cortex are shown in Text-fig. 4. These populations varied with the stage of decomposition and with the habitat. The most active population of mycelia originated from the root surface; as the discoloration of the cortex developed in intensity, so did the hyphal populations increase in size in all root habitats.



Text-fig. 4. Mycelial populations of roots at various stages of decomposition.

	Numbers of segments in samples: numbers of roots given in parentheses		
	Root surface	Outer cortex	Inner cortex
□ White roots, apices	730 (57)	109 (4)	109 (4)
▨ Light-brown roots	386 (30)	393 (23)	543 (38)
■ Dark-brown roots	240 (19)	30 (6)	30 (6)

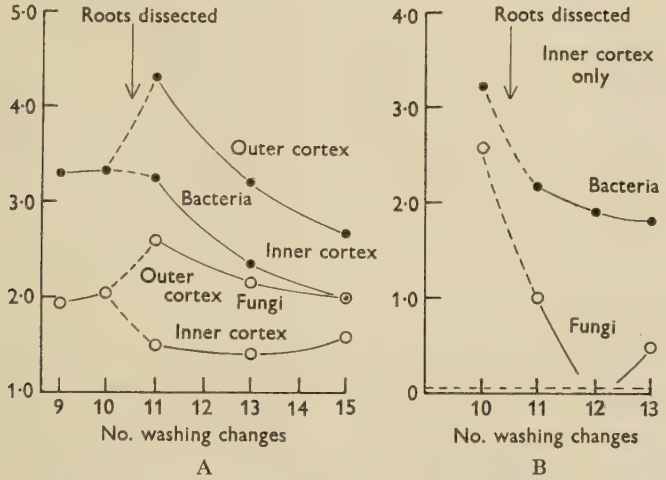
A, Semi-quantitative estimate of mycelial activity. Ordinate—index of growth activity per 100 segments sampled. B, Root fragments inhabited by fungi. Ordinate—percentage of segments inhabited by fungi. C, Total number of species of fungi. Ordinate—frequency per 100 segments sampled. D, Distribution of sterile mycelia. Ordinate—(1) percentage occurrence, (2) percentage total population (shown in dotted outlines).

These conclusions were arrived at in three ways. First, the most active population, as measured by the growth rate of mycelia, i.e. index of growth on nutrient agar, originated from the root surfaces. Activity progressively decreased in the populations derived from the outer and inner cortex (Text-fig. 4 A). The progress of root degradation was accompanied by an increase in activity in the fungi from each zone, though the extent of this increase was not so great as the gradient of activity across the cortex.

Microscopical examination of dissected roots showed that the outer cortex of white roots was only partially infected by mycelia penetrating from the root surface; on the other hand, both outer and inner cortex of brown roots were fully infected by fungi. This indicates that the cortical tissues of the brown roots had lost resistance to infection and were in the early stages of senescence; fungi had not, however, penetrated the endodermis.

Secondly, the percentage of root fragments which were inhabited by fungi (Text-fig. 4 B) increased with the age of the material, but the progress of colonization was from the root surface to outer cortex and to inner cortex.

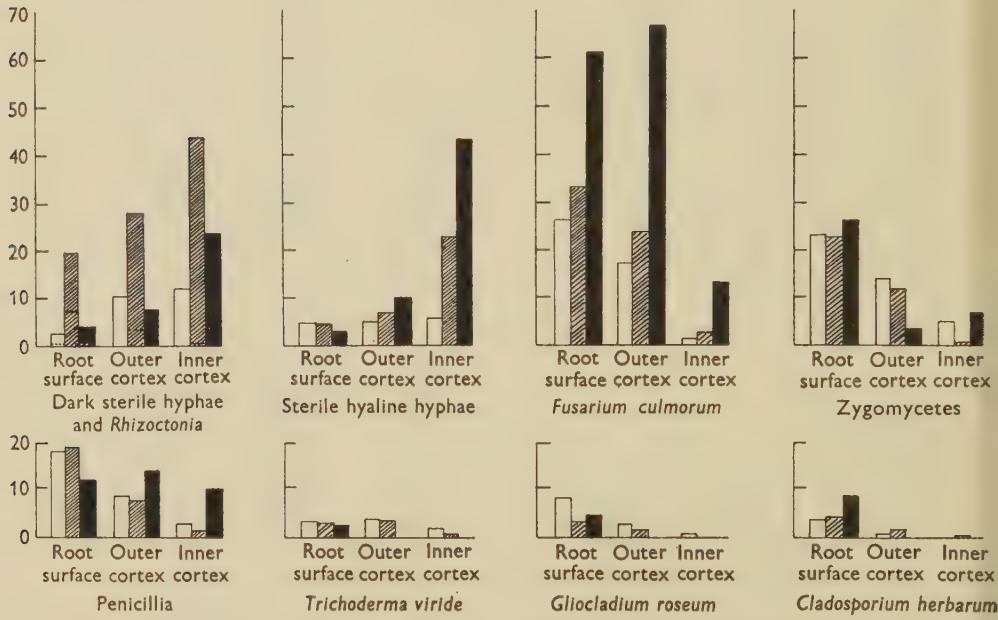
Finally, the total of all kinds of fungi, i.e. frequency per 100 segments, in each of the various root habitats at each stage of decay (Text-fig. 4 C) followed the same pattern.



Text-fig. 5. Influence of dissection upon numbers of fungi and bacteria in washings of light-brown roots. Ordinate—logarithm of number of micro-organisms (log 10 ×). ●, bacteria; ○, fungi. A, Counts are the total for a sample of five roots. All fragments were washed after dissection. B, Counts are the total for a sample of three roots. Only the inner root cylinder was washed after dissection.

The population of the outer cortex was smaller than that of the root surface, which was due to the loss of some sporing fungi during dissection. This point is brought out in the next histogram (Text-fig. 4 D) which demonstrates that the root surface flora contains fewer sterile forms than the cortex. Antagonists, as well as fungi which form dense mycelial growth in culture and prevent observation of other species, were reduced in number by the process of dissection, which permitted the development of more colonies of sterile mycelia from fragments of outer cortex. Two experimental observations, which confirmed the view that many sporing fungi were dislodged from brown roots when they were dissected, are shown graphically in Text-fig. 5. This figure shows that large numbers of micro-organisms occurred in the washings of the fragments of *outer cortex*

immediately after dissection (washing 11), which were undoubtedly due to the disruption of various sites of microbial activity in the root region during dissection. Washing change 15 of the fragments of outer cortex contained a lower proportion of bacteria to fungi, as compared with that in washings 9 and 10 of intact roots. It would seem that many colonies of bacteria were dislodged from the roots as a result of dissection, while the mycelia of fungi were closely associated with the root tissues. The washings of the fragments of the stelar cylinders contained fewer micro-organisms



Text-fig. 6. Distribution in root tissues of various species and groups of fungi. Ordinates—percentage occurrence. □, white roots, apices; ▨, light-brown roots; ■, dark-brown roots.

than those of the undissected roots, but the actual species of fungi contained in these washings were identical with those which developed from the plated fragments of the *outer sheath* of the cortex. Only one of the species of fungi determined in the washings of the root cylinder, a *Fusarium*, was found growing from fragments of inner cortex (once in a sample of 125 fragments) but this species was very common in the outer cortex. This shows that during root dissection the fragments of inner cortex were contaminated with fungi, and probably with bacteria, from the outer cortex.

Information about the composition of the floras of the various root habitats is given in Text-fig. 6. Slight differences in the distribution of the dark-coloured and the hyaline kinds of sterile mycelia can be recognized. The dark-coloured mycelia were very abundant in the outer cortex of white root apices and light-brown roots, which indicates that these fungi were early colonizers of these tissues.

Microscopical examination of light-brown roots revealed that brown hyphae of small diameter extended along the middle cortex (branching hyphae shown in Pl. 1, fig. 4). The hyphae were connected with small sclerotia, consisting of 5–30 segments, which occupied cortical cells. As many as three of these hyphae could be seen in transverse sections. The morphology of the isolated mycelium did not resemble that of *Rhizoctonia solani* Kühn, but it is probably an allied form.

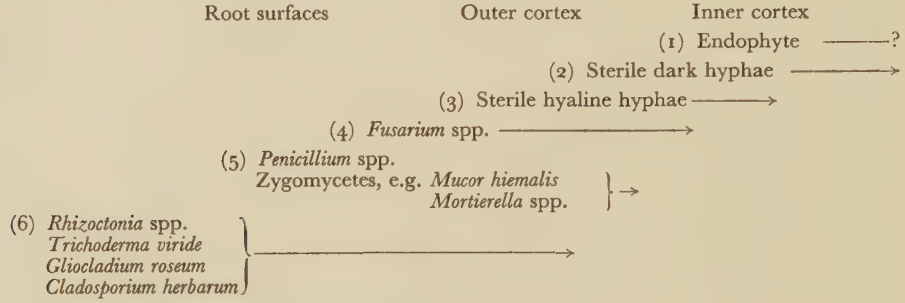
Sterile hyaline hyphae, which formed characteristic and slow-growing colonies upon nutrient agar, increased in abundance in the inner cortex as it aged. It seems then that the breakdown of the inner cortex is initiated by a range of sterile forms, which are undoubtedly underestimated by conventional techniques, because of their inability to compete in culture with more vigorous forms. It seems likely that they have an almost continuous mycelial existence in their 'natural' habitats.

The numbers of *Fusarium culmorum* increased with the degree of decomposition of the roots. This fungus was abundant on the root surfaces and in the outer cortex and appeared to have begun to colonize the inner cortex of older roots from without. The observation that saprophytic Fusaria are primary colonizers of decomposing tissues is in agreement with the findings of Samuel & Greaney (1937) who found that *F. culmorum* could be isolated in increasing numbers from the stubble of healthy wheat plants with lapse of time from harvest. Sadasivan (1939) found that Fusaria and *Mucor* spp. were more abundant than Penicillia in the early stages of decomposition of wheat straw buried in soil, but later the fungal population was dominated by Penicillia. The evidence presented here indicates that Fusaria dominated the mycelial population of the root surfaces to an increasing degree at each stage of the decomposition of the cortex, whereas the numbers of Zygomycetes and of Penicillia hardly altered. It may be recalled that Phycomycetes (apart from the endophyte) were only seen upon the surfaces of roots.

The distribution of *Trichoderma viride*, *Gliocladium roseum*, *Cladosporium herbarum* and *Rhizoctonia* spp. demonstrates that these were inhabitants of the root surfaces. Generally a fungus such as *Mucor*, *Penicillium* or *Fusarium* was not isolated from the inner cortex unless it had colonized several segments of the outer cortex in the same region of root. This suggests that these fungi were unable to infect the inner cortex of healthy roots until the outer cortex had decomposed. These fungi, excepting *Rhizoctonia*, generally overgrow other organisms in mixed culture and often produce antibiotics. They also have a high rate of spore production, which is achieved after a short period of hyphal activity. Such fungi probably exist as secondary saprophytes upon semi-decomposed substrates in competition with the pioneer flora of sterile forms of mycelia. Their period of hyphal activity is probably terminated by the exhaustion of the substrate, colonization of new substrates being initiated by the germination of propagules.

Discussion

The picture that emerges is that the cortex is not a simple substrate which is broken down by the activities of a wide range of soil fungi but that it is a region of varied habitats which have their own specialized floras (see Garrett, 1955). The succession of fungi in the cortex can be summarized as following a sequence, such as outlined below.



The results show agreement between the isolation-plate and direct methods of sampling in respect of the distribution of the inhabitants of the decomposing cortex. Unfortunately there is no evidence to show how far the decomposition is initiated either by the activities of semi-parasitic fungi or by autolysis within the cells of the cortex. It is clear, however, that in the early stages of fungal colonization there is a specialized flora which must overcome host resistance, but with the absence of competitors. These pioneer fungi appear to have a low degree of competitive saprophytic ability, since their behaviour is similar to the saprophytic behaviour of some other parasitic root-infecting fungi (Garrett, 1950). With senescence, host resistance declines and the environment becomes selective towards those fungi with a high degree of competitive saprophytic ability. As colonization progresses the cortex becomes occupied by competing saprophytes, which out-number the pioneer flora, and are less specialized in their demands upon the habitat.

This interpretation agrees with the findings of Lucas (1955), who studied the colonization of a dead plant tissue by two different fungi in competition with the soil microflora. The degree of colonization of wheat straw by *Ophiobolus graminis*, which is a root-inhabiting fungus of low competitive saprophytic ability, was less than that of a soil-inhabiting fungus, *Fusarium culmorum*, which has a higher degree of competitive saprophytic ability.

Throughout this survey no fungi were observed either penetrating the endodermis or within the steles of roots possessing a cortex, with the exception of diseased roots. This poses the question of determining the conditions under which fungi colonize the tissues of the stele. The steles of brown roots with intact cortices probably function as conducting strands and may possess a certain degree of resistance to infection. In such circumstances fungi will not penetrate the endodermis until there is a decrease in the physiological resistance of the stele tissues.

It is not known how, or in what way, the environmental conditions of the cortex determine the sequence of colonization. A study of conditions, such as gradients of nutrient supply, gas concentration, microbiological interaction and host-tissue resistance across the cortex, presents the practical difficulty of obtaining reliable measurements upon material of such small volume. An alternative, though less satisfactory, approach would be the study of behaviour of the isolates in pure culture, as a preliminary step to an appreciation of the influence of their natural environment. An account of such a study of the influence of limited oxygen supply upon growth and respiration of a few selected isolates will be given elsewhere.

I should like to thank Dr J. L. Harley for his guidance and interest throughout this work, Prof. C. D. Darlington for allowing the greater part of this investigation to be made at the Department of Botany, Oxford and Dr William Davies, Mr T. E. Williams and Mr G. Samuel for their encouragement of these studies. I am grateful to Mr A. H. Cripps for technical assistance and to all those who have helped me with the preparation of this manuscript, in particular Mr P. Turner and Mr J. Corrall.

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EXPLANATION OF PLATE 15

Fig. 1. Semi-decomposed root of ryegrass. T.S. stained in safranin and light-green, × 120. Cells of outer cortex have thickened walls, no intercellular spaces. Cells of middle cortex are decomposing, tangential walls have ruptured. Stele damaged in sectioning.

Fig. 2. Same as Fig. 1 but L.S., × 120.

Fig. 3. Dissected white root of ryegrass mounted in lactophenol, × *c.* 480. Hyphae of endophyte can be seen in cells of cortex adjacent to stele.

Fig. 4. As Fig. 1, but × 480. Shows branching hyphae of dark coloured mycelium in cells of middle cortex.

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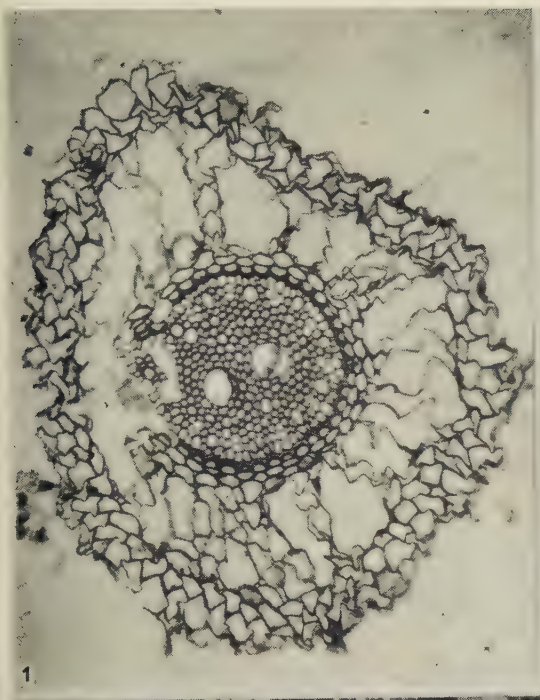

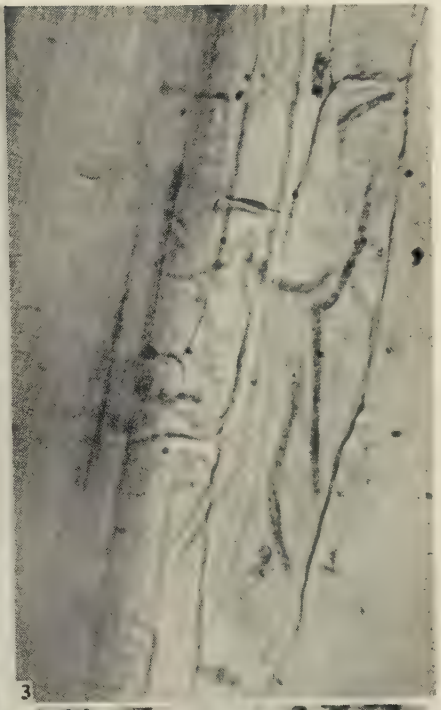

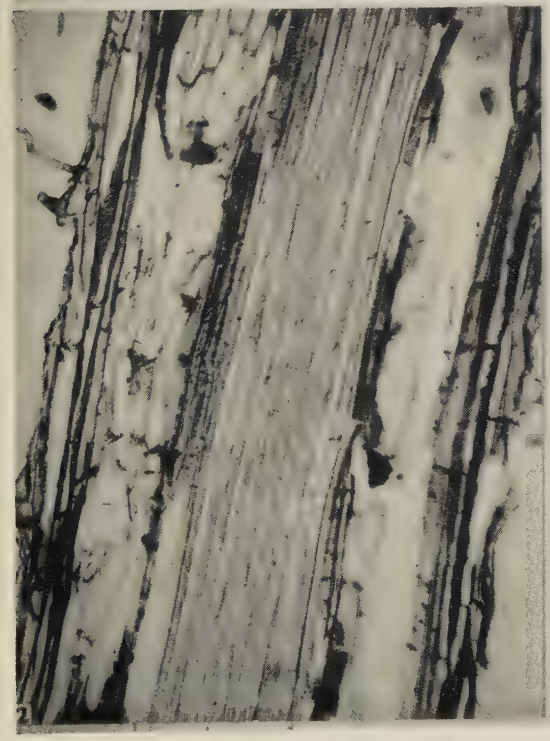

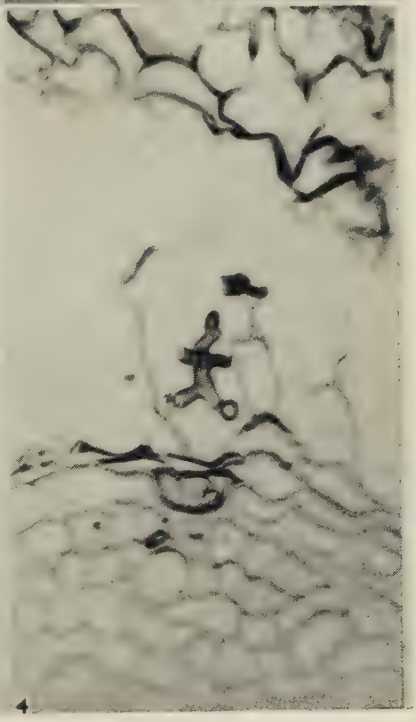

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Trans. Brit. mycol. Soc. **40** (3), 407–408 (1957)

HAUSTORIA OF *PHYTOPHTHORA ERYTHROSEPTICA* IN POTATO TUBERS AFFECTED WITH PINK ROT

By R. McKAY, J. B. LOUGHNANE and T. KAVANAGH

Department of Plant Pathology, University College, Dublin

(With Plate 16)

An account is given of the occurrence of haustoria of *Phytophthora erythroseptica* Pethybr. in potato tubers affected with Pink Rot.

In a recent article in these *Transactions* Blackwell (1953) defined two broad divisions of the *Phytophthoras*, i.e. a group possessing haustoria, and another group in which haustoria are absent. The present note records the occurrence of haustoria in *Phytophthora erythroseptica* Pethybr.

Pethybridge (1913, p. 537), in his original account of Pink Rot, states: 'No haustoria were observed entering the cells but special efforts to prove their presence or absence were not made.' Apparently, as a result of this statement, it has been assumed that haustoria are non-existent in this species (Butler & Jones, 1949). It is true that there is no description of haustoria of *P. erythroseptica*, which is rather surprising, in view of the common occurrence of haustoria in tubers affected with Pink Rot.

Owing to the rapid development of disease in potato tubers inoculated with *P. erythroseptica*, this fungus has been used for many years in class work at Glasnevin for demonstrating the practical application of Koch's Rules. Not only so, but with a little manipulation and a minimum expenditure of time and equipment, both sexual and asexual organs of the fungus can be easily obtained, their germination observed, and every stage in the life-history of the organism shown to students.

About five years ago, Loughnane drew the senior author's attention to the occurrence of haustoria of this species in potatoes. Since then, their presence has been verified annually during ordinary class work. At the same time, their development may be considered as somewhat sporadic, for they do not occur in every diseased tuber, even of the same cultivar. Nevertheless, when haustoria do develop they are often quite numerous, and more than one may be found in a single host cell (Pl. 16, fig. 1). Several different isolations of *P. erythroseptica* have been made from naturally occurring diseased tubers, and all have behaved in a similar manner.

Haustoria have been seen in tubers of the following cultivars: Arran Banner, Arran Pilot, Majestic and Up-to-Date. The haustoria are without any callose sheaths, and they may be simple finger-like processes or branched. Each is attached to its hypha by a slender stalk which passes through a minute opening in the cell wall, Pl. 16, fig. 2.

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EXPLANATION OF PLATE 16

Fig. 1. *Phytophthora erythroseptica*. Section of Up-to-Date potato tuber showing host cell with six haustoria, two of which are branched. × 140.

Fig. 2. Section showing attachment of haustorium to parent hypha by a very slender stalk which penetrates the host cell wall by a minute opening. × 320.

(*Accepted for publication* 5 *December* 1956)

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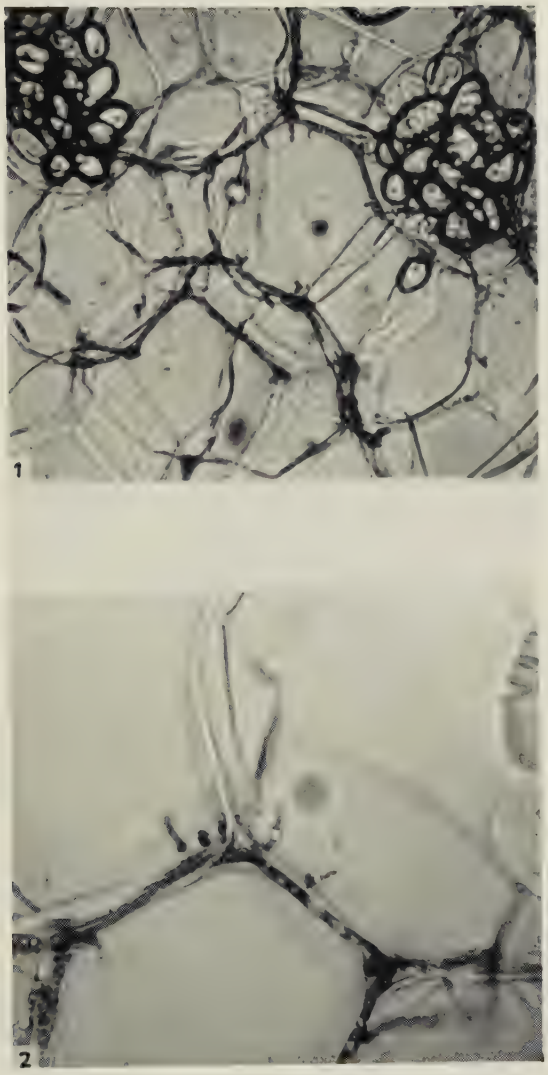

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Trans. Brit. mycol. Soc. **40** (3), 409–414 (1957).

A STUDY OF AIR-BORNE *CLADOSPORIUM*

By R. R. DAVIES

Biological Department, St Thomas's Hospital Medical School, London

(With 1 Text-figure)

The viability of air-borne *Cladosporium* was studied by means of a slit sampler. From the results obtained it was concluded that the numbers of non-viable *Cladosporium* dispersion units in the autumn air at London is insignificant. A series of air-borne spore samples, taken by means of a cascade impactor and a slit sampler, showed that there was little to choose between the relative efficiencies of the two methods.

Introduction

During September and October 1955, samples of the 'air-spora' were taken by means of a slit sampler* (Bourdillon, Lidwell & Thomas, 1941) and a cascade impactor (May, 1945) to compare the two methods. With the former, air is sucked through a narrow slit on to the surface of the agar in a Petri dish, and the number of air-borne organisms calculated from the number of colonies which subsequently develop. The cascade impactor, on the other hand, comprises a series of jets through which air-borne particles are impacted on to vaseline-coated glass slides. In this case the number of air-borne spores is calculated from the number recognized in the slide deposits. Clearly, the two methods will not necessarily give the same results, for the one counts only the viable reproductive units which develop sufficiently rapidly on the medium employed, while the other counts spores, regardless of viability.

Because the spores may be identified with relative ease, *Cladosporium* was used as the test organism. A number of workers, notably Gregory (1952), Hyde & Williams (1953), Hirst (1953) and Pady & Kelly (1953), have shown that *Cladosporium* spores occur in the air both singly and in clumps. A clump will behave as a single reproductive unit. Consequently, records were kept of the numbers of single spores and of the numbers of groups of various size (containing 2, 3, 4, 5 or more spores etc.).

The Viability of Air-borne *Cladosporium*

To investigate the ability of air-borne dispersion units of *Cladosporium* to germinate, plates of filtered Dox's agar were placed in the slit sampler and air sampled for various periods, with either the plate rotating, when

* The slit sampler used was constructed in this laboratory and differed from that described by Bourdillon *et al.* in several features. The slit measured 1 mm. × 30 mm. and the instrument was operated with an air flow of 20 l./min. For most efficient impaction, the distance between the slit and agar surface was reduced to a minimum, and in the experiments described, owing to difficulty in obtaining perfectly horizontal surfaces in Petri dishes, this distance varied between approximately 0·5 and 1 mm.

sampling was prolonged to obtain a sufficiently dense deposit for microscopic examination, or stationary when an easily examined rectangular trace was produced from a much shorter exposure. After incubation on the laboratory bench for various periods, the plates were examined under the microscope, and 100 dispersion units selected at random on each plate were examined for germination. The results are shown in Table 1.

Table 1. *Percentage germination of* Cladosporium *dispersion units on Dox's agar*

After 20–24 hr.	94, 88, 87, 96, 93, 88, 84, 87, 95, 95, 92, 92, 90, 64*, 32*, 36*
After 40–44 hr.	93, 43*, 96 Discontinued because by this time some colonies had begun spore production.

* Records from deposits containing large amounts of soot and ash.

The fact that a small percentage of dispersion units did not germinate within a limited period cannot be taken as evidence of non-viability, because some spores germinate more slowly than others, and the germination of spores in which germination is delayed may be entirely inhibited by the staling products of the more rapidly developing colonies. In this respect it must be emphasized that the samples did not only contain *Cladosporium*, and that the spores of other common air-borne moulds such as *Alternaria*, *Botrytis*, *Epicoccum*, *Penicillium* and *Pullularia* were present and also germinated to form colonies.

During the experiments it was noted that the presence of soot and ash on the plates appeared to inhibit spore germination. On 17 July 1956 at approximately 16.00 hr., there was a sudden change in weather conditions and a cloud of soot and ash occurred over Lambeth. During this period air was being sampled in the laboratory by a slit sampler, using stationary plates of Dox's agar. The trace deposits were dark to the naked eye. The 'smog' deposit on a trace containing the contents from 600 l. of air was so dense as to make spore counting difficult, and in this deposit only 32% of the *Cladosporium* dispersion units germinated after 20 hr. A lighter deposit showed 64% germination, whilst in another deposit, after 44 hr., 43% of the dispersion units had germinated.

A comparison of samples taken with a slit sampler and a cascade impactor

The sites chosen for sampling were a laboratory on the ground floor, and the 20 ft. high flat roof of St Thomas's Hospital Medical School overlooking the Albert Embankment. Only one vacuum pump was available and the procedure adopted was as follows:

The first plate was inserted in the slit sampler, the distance between the agar surface and the slit adjusted, and the air sampled at 20 l./min. for 3 min. After sampling the plate was at once removed and covered with a sterile lid. The pump was then connected to the cascade impactor and a 10 min. sample taken at 17·5 l./min. This procedure was then repeated with the difference that the slit sampler was run for 4 min. Finally a second 3 min. sample was taken with the slit sampler.

The over-all samples consisted of the contents of 350 l. of air impacted on to one set of cascade impactor slides, and the contents of 200 l. of air trapped on three plates of 2 % malt extract agar.

The total sampling period outdoors extended over 40–45 min., as also did the indoor sampling which was usually commenced about 30 min. later. Indoors both instruments were used with their orifices facing upwards, but outdoors, whereas the orifice of the slit sampler faced upwards, that of the cascade impactor was directed into the wind. In so far as it was possible, sampling was carried out on alternate afternoons from 15.00 hr. onwards.

The plates were incubated on the laboratory bench, and the number of *Cladosporium* colonies which developed after 5 days counted; this total from each 3-plate sample was multiplied by 5 to obtain an estimate of the numbers of dispersion units per cubic metre.

The cascade impactor slides were prepared for use, and finally mounted in Solvar as recommended by Prof. P. H. Gregory (personal communication), which represents a slight modification of the method used by Hirst (1953). The spores and clumps in one-tenth of the total trace areas were counted in traverses at right angles across the deposit, and the results expressed as dispersion units per cubic metre. The results are summarized in Fig. 1 and Table 2. In a statistical analysis of the results obtained outdoors, the mean of the twenty-eight estimates of dispersion units obtained with the slit sampler was compared with that from the cascade impactor by using Student's *t*-test. It was shown that for $P = 0{\cdot}01$ the difference between the means was insignificant. The same test was applied to the indoor data and the same result obtained.

Table 2. *The sums of the twenty-eight estimates obtained by each of the two methods*

	Indoors	Outdoors
Cascade impactor		
Sum of spores	15,584	42,868
Sum of dispersion units	11,754	24,402
Slit sampler		
Sum of dispersion units	11,617	22,378

Bearing in mind that the distribution of *Cladosporium* in air may vary during even short intervals of time, the results obtained by the two methods show a very good degree of agreement. Outdoors, however, on two occasions there were very large differences; on 19 September, the estimate obtained with the cascade impactor was nearly twice that with the slit sampler, while on 10 October the slit sampler estimate was approximately three times that of the cascade impactor. The fact that throughout the period of the investigation neither sampler gave a consistently higher estimate than the other, makes it likely that these differences were due to variations in the actual numbers of dispersion units during the sampling periods.

Indoors, the only large discrepancy between the two sets of results is that shown on 14 September, when the slit sampler estimate was nearly twice as high as that made with the cascade impactor. On this occasion

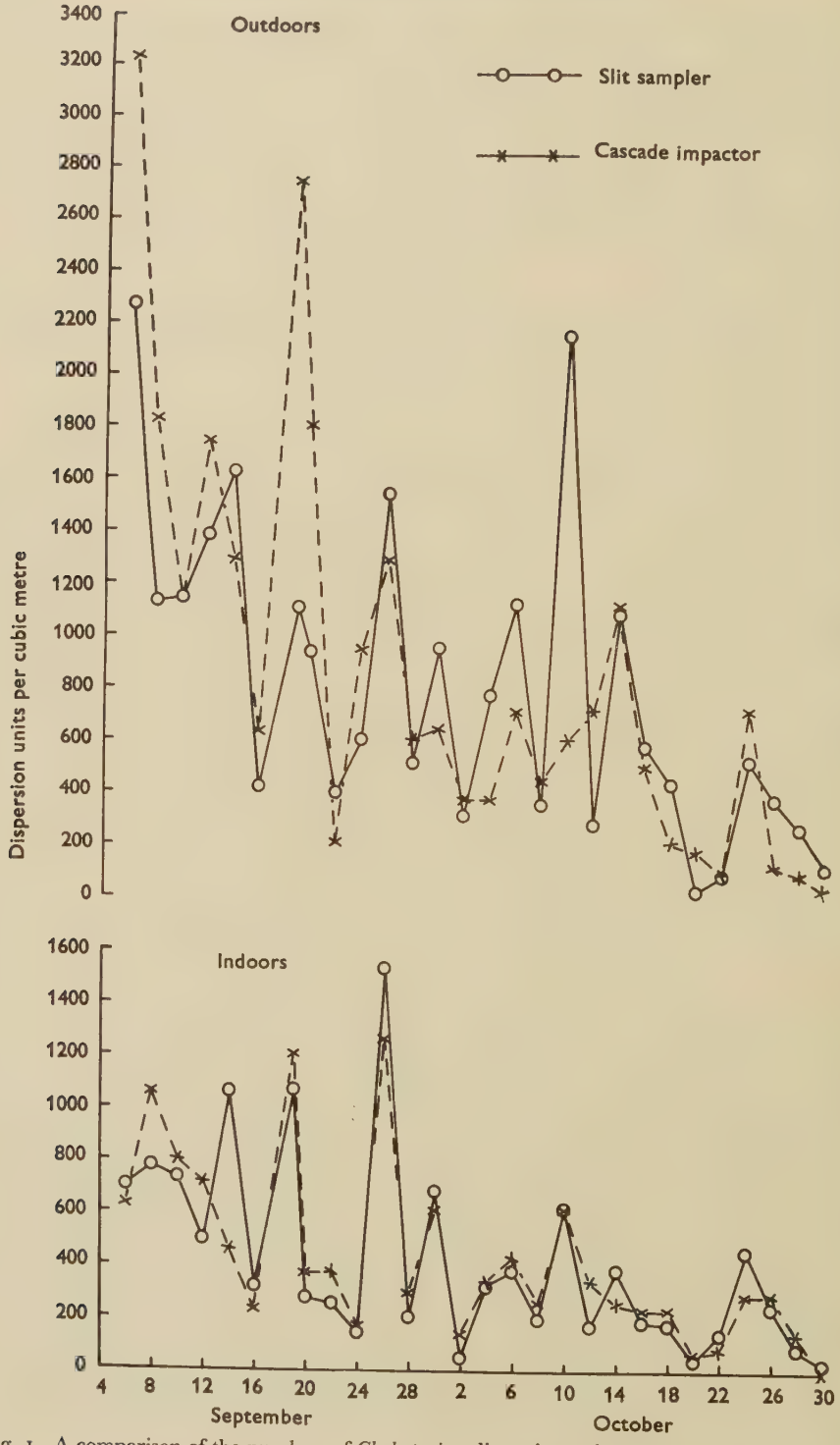

Fig. 1. A comparison of the numbers of *Cladosporium* dispersion units per cubic metre estimated by means of a slit sampler and a cascade impactor on twenty-eight afternoons during September and October 1955.

the numbers of colonies which developed on the plates exposed in the slit sampler were:

Plate (i) 15.12–15.15 hr. from 60 l.	91 colonies
Plate (ii) 15.28–15.32 hr. from 80 l.	71 colonies
Plate (iii) 15.47–15.50 hr. from 60 l.	50 colonies

which shows that the numbers of air-borne dispersion units diminished during the total sampling period.

A study of the dispersion units collected with the cascade impactor given in Table 3 not only shows the numbers of spores which comprise the typical units, but also gives a good indication of the numbers of colonies which develop from single spores, and groups or clumps containing various numbers of spores. From these results it appears that the *Cladosporium* cloud outdoors contains a higher percentage of spore groups, and larger clumps than that indoors, although statistically it cannot be shown that these differences are not due to chance at the 0·05 level.

Table 3. *Analysis of the total dispersion units counted in the samples taken with the cascade impactor during September and October* 1955

	Indoors		Outdoors	
		%		%
Single spores	329	81·43	571	69·63
Groups of 2 spores	52	12·87	128	15·55
Groups of 3 spores	15	3·71	51	6·19
Groups of 4 spores	2	0·49	31	3·76
Groups of 5 or more	6	1·48	40	4·86

Discussion

The results given above differ from those of Hyde & Williams (1953), who state 'nine-tenths of the conidia arrived on our slides in groups (of up to 100) and nearly one-half either as single conidia or in groups of two to six', and that their slides exposed at Cardiff in June and July 1949 '. . . showed that 4286 units of dispersal comprised in all 15,553 conidia, an average of 3·62 conidia per unit'. An explanation of these differences might be that of Hirst (1953), who states that freely exposed surface traps are highly selective and favour the heavier spores. It appears that the 'gravity-slide method' of Hyde & Williams overestimates the numbers of air-borne spore clumps because of the greater rate of deposition of the larger units. From data given by Gregory (1954), when two spore traps were operated simultaneously in a 40 ft. tower, I calculated the average dispersion unit comprised 2 spores, a figure comparable to that of 1·7, derived from estimates in Table 2 (impactor, outdoors).

The study of the viability of air-borne *Cladosporium* described above shows that, of 1500 dispersion units examined, 91 % were germinated within 44 hr. The fact that a number of dispersion units did not germinate within this period cannot be taken as evidence of non-viability (see p. 410). These results, together with the close correlation between the numbers of air-borne dispersion units estimated with the cascade impactor and those

estimated with the slit sampler, lead to the conclusion that it is probable that the number of non-viable *Cladosporium* dispersion units in the autumn air at London is insignificant. This conclusion differs from that of Pady & Kapica (1955), and Pady & Kelly (1953), who studied the viability of *Cladosporium* spores in air by comparing the number of spores on silicone slides exposed in a slit sampler with the number of colonies which developed in plates exposed in a McGill-GE sampler. Pady & Kapica (1955), in a flight between London and Prestwick on 29 August 1951, calculated from their silicone slide study that the air contained 16·3 *Cladosporium* spores per cubic foot, while their plate study gave 0·4 per cubic foot; they concluded that there is a loss in viability of spores while they are air-borne. In a study of arctic air, Pady & Kelly (1953) stated that the highest estimates of air-borne *Cladosporium* spores which they calculated from colonies which developed on plates was 1·9 per cubic foot, whereas on silicone slides a concentration of 8·2 per cubic foot was recorded. Pady and associates, however, noted that the *Cladosporium* spores were present in the air in groups, and realized a group would initiate a single colony; nevertheless they appear to have compared the numbers of colonies which developed in their plates with the total numbers of spores on their slides, and not with the numbers of dispersion units. Furthermore, Pady & Kapica (1955) report that during their flights there were variations between the samplers even when sampling the same air, and that the slit sampler gave readings that were two to eight times higher than the McGill-GE sampler.

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THE POSSIBLE SIGNIFICANCE OF TOXIC METABOLITES OF *VERTICILLIUM ALBO-ATRUM* IN THE DEVELOPMENT OF HOP WILT SYMPTOMS

By P. W. TALBOYS

East Malling Research Station

(With Plate 17 and 7 Text-figures)

Culture filtrates of *Verticillium albo-atrum* will induce desiccation and necrosis in hop shoots, but the intensity of this action bears no relation either to the pathogenicity of the fungal strain or to the wilt tolerance of the hop variety.

Considering this in relation to evidence from earlier work with intervarietal graft complexes, it is suggested that, in a *determinative* phase of the host-parasite relationship, the interactions of the mechanisms which determine the degree of tolerance of the host and the pathogenicity of the fungus lead to the establishment of varying intensities of vascular invasion in the root system. In a secondary *expressive* phase, continued but sometimes restricted fungal activity results in development of visible symptoms, possibly through a toxigenic mechanism in which symptom intensity depends on toxin dosage and is related to the amount of mycelium present in the vascular system.

Introduction

Two distinct forms of *Verticillium* wilt of the hop (*Humulus lupulus*) have been recognized by Keyworth (1942); the first, widely distributed in hop gardens, is a mild disorder (known as 'fluctuating wilt'), in which there tend to be fluctuations from year to year both in the number of plants showing symptoms and in the severity of the symptoms; the second is a severe and generally lethal disease ('progressive wilt') at present known only in the south-east of England, where it appeared initially in the early 1930's. Isaac & Keyworth (1948) have shown that the two types of outbreak result from infection by strains of *Verticillium albo-atrum* differing in pathogenicity to the hop, but morphologically indistinguishable in pure culture.

In the course of investigations on the control of the disease in its acute form, a wide range of existing commercial varieties and of new seedling varieties bred at Wye College, Kent, has been tested for resistance to the disease (Keyworth, 1947; Keyworth, Hitchcock & Goode, 1953). A number of the seedlings has shown a high degree of tolerance to the pathogen, but none has proved to be immune.

Present studies of the mechanisms underlying differential tolerance in the host and differential pathogenicity in the parasite include an examination of the processes leading to the development of the characteristic wilt syndrome described by Keyworth (1942).

There is a great divergence of opinion on the mechanisms by which

vascular mycoses induce desiccation of the leaves and stems of the host, and it has been customary to refer to two main types of theory: in the first, it is suggested that toxic metabolites secreted by the pathogen, and distributed in the transpiration stream throughout the aerial parts of the plant, damage its leaf tissues and so destroy its water-regulating mechanisms; and in the second, that mechanical blockage of the vessels occurs, and causes an acute water deficit in the stem and leaves. This dichotomy appears at the present time to represent a considerable over-simplification of the problem, and it is not profitable to attempt to classify contemporary theories within such a grouping.

The case for considering 'systemic toxaemia' as the primary cause of wilting has been discussed in detail by Gäumann (1951) with special reference to lycomarasmin, a peptide produced by *Fusarium oxysporum* f. *lycopersici*. In a general discussion on action of toxins in plant diseases, Dimond & Waggoner (1953*b*) have emphasized that though many pathogens produce, in culture, metabolites which are toxic to plants (and are therefore described as 'toxins'), it must not be assumed that such substances are active *in vivo*. They propose the term 'vivo-toxin' to refer to a substance, produced in the infected host by the pathogen and/or its host, which functions in the production of disease, but is not itself the direct inciting agent.

Dimond & Waggoner (1953*a*) had shown that lycomarasmin could not be regarded as a true vivo-toxin. The term could, however, be applied to the toxic substances extracted by Gottlieb (1943) from the vascular system of *Fusarium*-infected tomato plants. These substances were reported (Gottlieb, 1944) to cause an increase in host-cell permeability. In studies of cotton wilt, caused by *F. vasinfectum*, Lakshminarayanan & Subramanian (1955) have demonstrated the vivo-toxic nature of fusaric acid.

The development of wilt symptoms in scions of various genera of Solanaceae on *Fusarium*-infected tomato rootstocks, before the fungus appeared above the graft, is presented by Davis (1954) as evidence for the action of toxins in advance of the pathogen.

In work on Dutch elm disease (*Ceratostomella ulmi*) Dimond (1947), and Dimond, Plumb, Stoddard, & Horsfall (1949) have shown that a number of substances may contribute to the phytotoxic action of the pathogen's metabolites, and have indicated the possible importance of a polysaccharide as a wilt-inducing agent. However, Feldman, Caroselli & Howard (1950) have concluded that though the polysaccharide *will* induce wilting, it is not of primary importance, and that an alcohol-soluble fraction of the culture filtrate is of greater significance in the development of this disease.

A wilt-inducing polysaccharide, apparently causing mechanical obstruction of the vessels of the tomato plant, has been isolated by Thomas (1949) from culture filtrates of *Fusarium solani* f. *eumartii*.

Hodgson, Peterson & Riker (1949) have shown that a number of polysaccharides could induce wilting in tomato plants; those that were soluble and giving clear solutions in water caused leaflet wilting, whereas those less soluble, giving opalescent solutions, caused flaccidity of stems and petioles. In the leaflet-wilting group, there was apparently a direct relationship between molecular weight and wilt-inducing capacity. Toxicity

was ascribed to mechanical obstruction of various parts of the vascular system of the plant.

Wilt-inducing polysaccharides have also been reported from various phytopathogenic bacteria (Feder & Ark, 1951), and from *Verticillium*, (Porter & Green, 1952).

Vascular obstruction was considered by Scheffer and Walker (1953) to be the primary cause of wilting in *Fusarium*-infected tomato plants; Ludwig (1952), in an extensive study of the same disease, concluded that such obstruction by a hyaline substance, visible only in fresh material, was sufficient to account for symptom development.

Thirty years earlier Bewley (1922) had observed a brown gum-like substance obstructing the vessels in fresh material of *Verticillium*-infected tomato plants. He suggested that the fungus secreted an exo-enzyme capable of producing wilt, either by direct action, or indirectly by inducing gum formation by the host.

Considerable attention has recently been paid to the possible role of pectic enzymes, and it has been suggested by various workers that, in the development of tomato wilt symptoms, a significant part is played by pectin methylesterase acting alone (Winstead & Walker, 1954*a*), with a pectin depolymerase (Gothoskar, Scheffer, Walker & Stahmann, 1955), or with a polygalacturonase (Waggoner & Dimond, 1955). Polygalacturonase with small amounts of pectin methylesterase have been identified by Scheffer, Gothoskar, Pierson & Collins (1956) in *Verticillium* culture filtrates.

It is generally considered that breakdown products resulting from the histolytic action of the enzymes accumulate in the vessels and obstruct the flow of water; confirmatory anatomical evidence has been put forward by Pierson, Gothoskar, Walker & Stahmann (1955).

However, Kamal & Wood (1955) are of the opinion that in *Verticillium* wilt of cotton the pectic enzymes of the fungus are not primarily responsible for symptom development.

Beckman, Kuntz, & Riker (1953) suggest that weakening of the structure of bordered pits by the action of pectinases might precede the development of the tyloses which cause vascular obstruction in the wilt disease of oak, in which the pathogen is *Chalara quercina*.

The action of *Fusarium* metabolites in stimulating the formation of obstructive tyloses in the vascular system of the sweet potato has been postulated by McClure (1950).

Waggoner & Dimond (1954) have examined the possibility that pathological wilting in the tomato results simply from obstruction of the vessels by mycelial masses. They conclude that though the physical presence of the fungus must contribute to the large decrease in flow resulting from infection, it cannot be the direct cause.

The present paper is concerned with an attempt to assess the role of *Verticillium* metabolites in the development of 'fluctuating' and 'progressive' wilt syndromes in the hop.

Methods

Excisions from the vascular cylinder in the lower part of the stems of wilt-infected plants readily yielded pure cultures of *V. albo-atrum*. These were maintained on 0·5 % prune extract agar augmented with 0·5 % lactose and 0·1 % 'Difco' yeast extract.

In the production of *Verticillium* metabolites for experiments, the fungus was grown in shallow culture in a liquid medium of the following composition: 1·0 % dextrose, 0·20 % potassium nitrate, 0·05 % potassium dihydrogen phosphate, 0·02 % magnesium sulphate, 0·001 % ferric chloride, 0·10 % 'Difco' yeast extract, distilled water to 100 %. The medium was dispensed in flat medicine bottles at a rate of 40 ml. per bottle, inoculated with a heavy spore suspension, and incubated at 22·5° C. for 10 days. At the end of this period the fungus had formed a thick gelatinous mat, and as much liquid as possible was removed by straining through muslin. Further filtration under pressure through a thick pad of paper pulp yielded a clear, faintly yellow solution free from fungal spores and mycelial fragments. Such solutions were used directly in tests to be described.

During the period of growth of the *Verticillium*, the composition of the culture medium was appreciably altered, and it was considered unsatisfactory to use the complete medium in control experiments to determine the effects on the plants of the residual components of the original medium. Analysis of the culture filtrate showed that the phosphate content was almost unchanged, that the nitrate concentration fell to about one-fifth of the original, and that the amount of residual dextrose was negligible. A control solution was therefore prepared similar in composition to the culture medium but with dextrose absent and potassium nitrate reduced to 0·04 %.

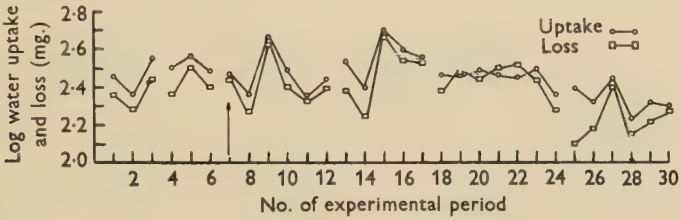
The method devised for studying the effects of *Verticillium* toxins on the water relations of detached hop shoots has been described in detail in an earlier paper (Talboys, 1952). Water uptake of a shoot enclosed in a bell-jar is determined by means of a calibrated potometer; a stream of dry air removes water lost in transpiration and passes it to sulphuric acid absorption tubes for estimation by weight. Estimations were made at hourly intervals for a minimum of 3 hr. on each of 4–7 days, depending on the rapidity of wilting.

The potometer was designed to permit rapid replacement of the water by test solutions of fungal metabolites without disturbing the shoot. In all experiments the plant was inserted in the apparatus and allowed to take up distilled water for at least 24 hr. before toxin treatments were started. In each diagram shown below, the time at which the test solution was added is indicated by a vertical arrow.

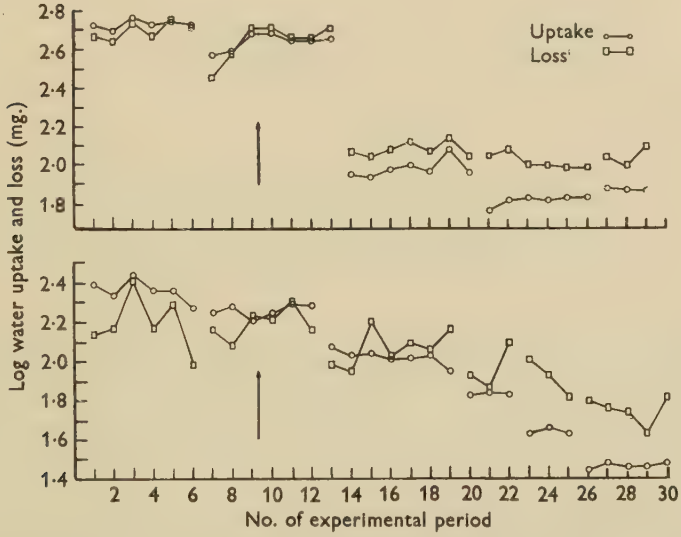
Two hop varieties have been used throughout the experiments, viz. 'Fuggle', a wilt-sensitive variety, and 'Keyworth's Midseason' (OR/55), which shows considerable wilt-tolerance.

Experimental results

In preliminary tests it had been found that shoots of tomato and hop standing in tubes of crude culture filtrates wilted, showed progressive desiccation, and died within a few days. Shoots standing in the control solution or in water remained alive and showed only slight chlorosis of the lower leaves after 6–7 days. The wilt-inducing capacity of the culture filtrates was not destroyed by boiling; it was therefore concluded that the substances concerned in the wilting process were not enzymes.



Text-fig. 1. Effect of a 'control' solution on the water balance of a shoot of OR/55.

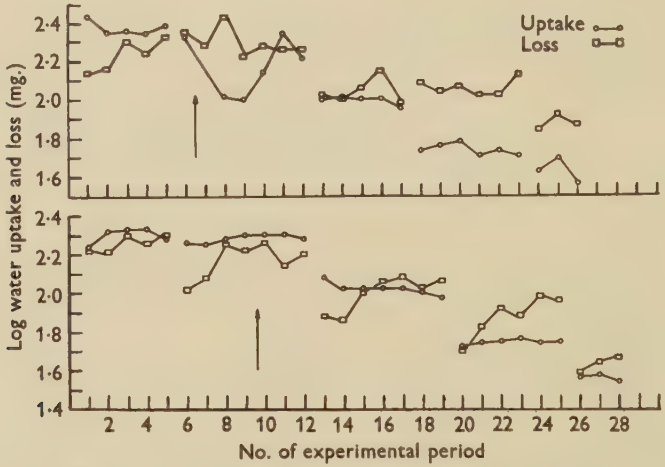


Text-fig. 2. Effect of a crude culture filtrate of a '*progressive*' isolate of *V. albo-atrum* on the water relations of shoots of OR/55 (above) and Fuggle.

The effects of the control solution and of the crude filtrates on the water relations of hop shoots are illustrated in Text-figs. 1–3. In each experiment it was apparent that the amounts of water taken up and lost before the application of the test solution (i.e. while the shoot was supplied with distilled water) corresponded very closely, though in general slightly more water was taken up than was lost. Text-fig. 1 shows that the addition of a control solution caused no change in this state of balance in a shoot of OR/55; at the end of the experiment the only symptom which had

developed was some chlorosis of the lower leaves. Similar results were obtained with shoots of Fuggle. The considerable fluctuations in the recorded values of both uptake and loss are probably attributable to variations in temperature and light intensity, which were uncontrollable in the laboratory.

The results of applying a culture filtrate of a highly pathogenic ('progressive') isolate of *V. albo-atrum* to shoots of OR/55 and Fuggle are shown in Text-fig. 2. In both varieties water uptake was substantially reduced, and though the transpiration rate fell also it did not become sufficiently small to restore a balanced condition. The leaves became desiccated and necrotic.



Text-fig. 3. Effect of a crude culture filtrate of a '*fluctuating*' isolate of *V. albo-atrum* on the water balance of shoots of OR/55 (above) and Fuggle.

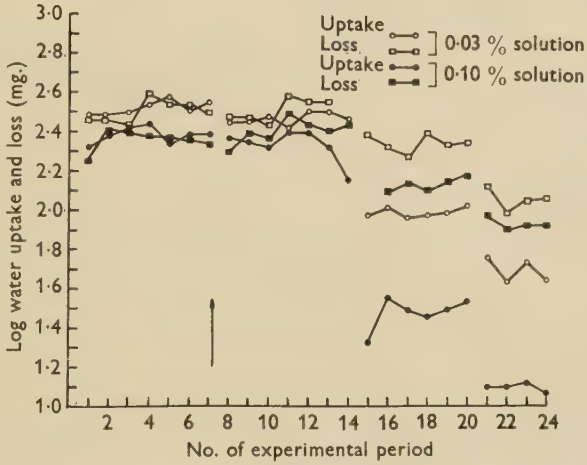
The results of similar experiments with culture filtrates from a mildly pathogenic ('fluctuating') isolate of the fungus are shown in Text-fig. 3. Again, in both varieties, the test solution induced a severe disturbance in water balance, water uptake was reduced much more than transpiration loss, and desiccation, upcurling and death of the leaves took place.

All of these tests were repeated at least once, and in every instance the results were substantially the same, though there was some slight evidence that culture filtrates from both isolates caused a more acutely unbalanced condition in the tolerant variety, OR/55, than in the sensitive Fuggle. This may have been due to chance variations in the physiological condition of the hop shoots and in the toxic capacity of the test solutions. However, it was found possible to precipitate the toxic material from the culture filtrate with ethanol and store it in a dry condition, thus enabling standardized toxin solutions to be prepared.

Ethanol (96 %) was added to the crude culture filtrate to give a final concentration of 60–65 % ethanol. The precipitate was removed by centrifuging, washed three times in 100 % ethanol and twice in ether, and then allowed to dry in an incubator at 25° C. The precipitate was powdered

and stored in a stoppered bottle. The yield amounted to 0·2–0·3 g. per litre of culture filtrate. Several batches were prepared and mixed thoroughly together before further tests were begun.

To determine whether the precipitate would induce the same reactions as the original filtrates, solutions at concentrations of 0·03 and 0·10 % were tested against shoots of the Fuggle variety. The results are shown in Text-fig. 4.



Text-fig. 4. Effect of two concentrations of precipitated toxin on the water balance of shoots of Fuggle.

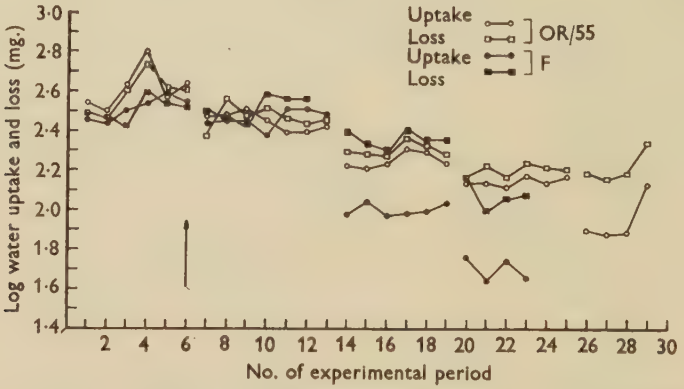
In both tests the reactions of the shoots followed the same general course as in previous experiments, but it was apparent that the higher concentration of toxin induced a much more severe reduction in water uptake. Water loss was affected to a smaller extent, so that a very acute water deficit occurred at an early stage, and rapid desiccation of the shoot took place. This relatively small effect of increased toxin concentration on transpiration is probably related to the fact that in a non-xerophytic leaf a considerable amount of water can still be lost through the epidermis and cuticle, even if the stomatal mechanism of control is undamaged and is operating to its fullest extent.

A solution of 0·03 % of the precipitated material was subsequently tested against a shoot of the tolerant variety, OR/55. The response of this, compared with that of the Fuggle shoot treated at the same concentration, is shown in Text-fig. 5.

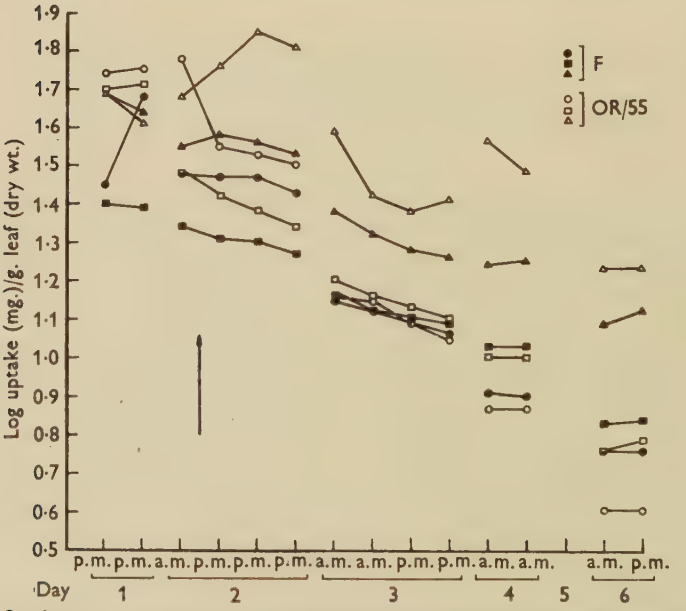
It is obvious that here the earlier suggestion of greater sensitivity of the tolerant variety was not upheld; the sensitive variety responded more rapidly and more acutely than the tolerant.

During the course of these experiments it became clear that the factor most closely related to the development of symptoms was the water uptake; transpiration tended to fall with the initial drop in uptake and then to remain fairly constant until desiccation was considerably advanced. It was therefore decided to determine water uptake alone in further tests, thus allowing a greater degree of replication.

Text-fig. 6 illustrates the result of one such test, in which three shoots of each variety were set up in simple potometers, and after 24 hr. in water were treated with an 0·03 % solution of a *Verticillium* toxin preparation.



Text-fig. 5. Effects of 0·03 % toxin solution on shoots of OR/55 and Fuggle.



Text-fig. 6. Water uptake of three shoots each of OR/55 and Fuggle, treated with 0·03 % toxin solution.

Each estimate of water uptake represents the average of three determinations taken over consecutive 5 min. periods; these were made on two or more occasions each day.

In this and other similar experiments, no consistent difference between the two varieties in respect of their response to the fungal metabolites could be found.

The stems of a number of shoots which developed acute symptoms under treatment were examined anatomically to see whether vascular obstruction could be considered a primary cause of wilting. Evidence of some obstruction was found in most specimens; a number of vessels contained a granular deposit, and tyloses were present in others. The proportion of vessels put out of action by obstructions appeared to be very small in most of the specimens, and it seemed improbable that they contributed significantly to the development of the unbalanced water economy in the shoots. No damage to the structure of the vessels or of their neighbouring tissues was observed.

The relation of induced symptoms to field symptoms

Throughout the experiments described above, treatment with *Verticillium* metabolites has invariably caused a progressive desiccation and necrosis of the leaves, but, except in a very few tests, the pattern of experimentally induced symptoms has differed considerably from that typically developed by *Verticillium*-infected plants in the field.

In progressive wilt, some initial yellowing of the leaves is followed by necrosis and upward curling of the margins. The dark brown, sharply defined, necrotic zone then extends along the interveinal areas towards the petiole. The veins remain green until necrosis is well advanced, and a characteristic striped appearance is thus produced, (Pl. 17, fig. 1). Finally the whole leaf becomes necrotic, and abscission takes place.

In the 'fluctuating' type of wilt, where symptoms are normally mild, leaf necrosis is generally limited to wedge-shaped sectors which are bounded by veins and are usually surrounded by a distinct chlorotic zone (Pl. 17, fig. 2). These symptoms often occur in a 'one sided' manner along a bine.

Though toxin-treated shoots have not shown symptoms closely resembling either of the natural syndromes, their appearance has tended to approach the 'progressive' type. The first visible response is a slightly water-soaked appearance in the interveinal areas, and this is followed by desiccation and upward curling of the leaf margins. Though these tissues are killed, they do not generally become dark brown, as in the field, but instead develop a dull brownish green coloration. The necrotic zone spreads inwards towards the petiole (Pl. 17, fig. 3), and, though there is a tendency for it to extend most rapidly between the main veins, this does not generally give rise to a striped appearance. The leaves finally become completely necrotic and frequently fall off after forming abscission tissues.

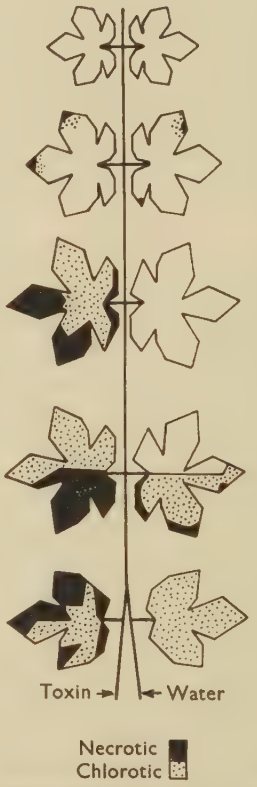
It has been found that the field symptoms are most closely simulated when the experimentally induced symptoms develop relatively slowly. This suggested that more satisfactory results might be obtained with more dilute toxin preparations. However, it was impracticable to test this, as periods of treatment exceeding 6–7 days resulted in chlorosis of the lower leaves of shoots in a control solution.

It is of interest to note that under glasshouse conditions the symptoms expressed by *Verticillium*-infected plants sometimes show considerable abnormalities. They have ranged from a fairly typical necrotic striping to a condition in which the leaves have become flaccid and have desiccated

very rapidly, with little change of colour. A number of plants have shown intermediate symptoms (Pl. 17, fig. 4) closely resembling those induced experimentally.

Similar symptoms have also been seen on occasional plants in experimental plots where the concentration of *Verticillium*-infected debris in the soil has been exceptionally high.

In none of the experiments which has been described did toxin-treated shoots develop the sectorial necrotic pattern characteristic of the mild disease. Limited damage of this kind has been attributed to a corresponding localization of the pathogen in the vascular cylinder, so, to obtain a localized distribution of toxin in a series of hop shoots, each stem was divided by a median longitudinal cut passing through the lowest node at right angles to the plane of the petioles. One half was then immersed in 0·03 % toxin solution, the other half in water. Text-fig. 7 illustrates diagrammatically the distribution of necrotic and chlorotic tissues which resulted in one such shoot. This distribution was comparable with symptoms developed in the 'fluctuating' type of wilt, though here again the necrotic zones were of a dull brown-green appearance, rather than dark brown, as in a *Verticillium*-infected plant.



Text-fig. 7. The pattern of chlorosis and necrosis induced in a hop shoot by one-sided application of a toxin solution.

Discussion

Experiments of the kind which have been described cannot *prove* that fungal toxins play a part in the development of wilt symptoms, unless it can be shown that the substances produced in culture occur also in the infected plant. Dimond & Waggoner (1953*b*) have pointed out that to prove that a substance is a vivo-toxin, it is necessary to fulfil a modified form of Koch's Postulates.

Nevertheless, having their limitations in mind, the experiments can provide useful circumstantial evidence, and it is in this light that they must be considered. They have shown that metabolites of *V. albo-atrum* produced in culture can induce desiccation and necrosis in hop shoots; though the experimentally induced symptoms do not closely resemble those normally found on diseased plants in the field, the fact that slow symptom development in toxin-treated shoots and unusually rapid symptom development in *Verticillium*-infected plants results in overlapping of the 'natural' and the experimentally induced syndrome types suggests that the differences may be related to the rate at which toxins accumulate at the site at which they act.

This site of action has not been exactly determined. The experiments

indicate that desiccation results from reduced water uptake rather than from excessive water loss, and suggest that the fungal metabolites play an obstructive role. However, the amount of vessel blockage observed in the stems and petioles appeared to be quite inadequate to account for such a great reduction in water uptake, and it seems probable that the action is effected mainly in the small vessels and tracheids forming the terminal elements in the vascular system of the leaf. Direct occlusion of the lumina of the vessels, or some process inhibiting the transfer of water from the vascular elements to the adjacent parenchymatous cells, could result in the desiccation of those cells.

It was found that the *intensity* of the toxin-induced syndrome was not related to the pathogenic potentialities of the fungal strain, nor to the wilt tolerance of the hop variety.

If the accumulation of such metabolites, either from virulent or from mild strains of *Verticillium*, will cause desiccation and necrosis in the leaves of both sensitive and tolerant varieties of the host, it is apparent that differences in the intensity of symptom expression cannot simply be interpreted in terms of varying degrees of toxin-sensitivity in the aerial parts of the host and of toxigenicity in the parasite; they could instead be attributed to factors which determine the quantity and distribution of the fungus within the plant, and which thus modify the quantity of the toxic substances produced.

Contributory evidence that the aerial parts of the plant do not play a determinative role in symptom development is provided by the grafting experiments of Keyworth (1953) and of Talboys & Wilson (1956). These experiments have shown that stems of both sensitive and tolerant varieties grafted to root systems of a sensitive variety develop acute symptoms when subjected to root infection by a virulent strain of *V. albo-atrum*, whereas mild symptoms are developed irrespective of the stem variety when the infected root system is of a tolerant variety; i.e. given the same conditions of root infection, the shoots of varieties of different tolerance respond in the same way.

Talboys & Wilson (1956) have pointed out that by the time symptoms in the aerial parts of the plant have given visual evidence of infection, the disease has already progressed to a stage beyond that at which the factors determining the intensity of the symptoms are operative. Thus it is possible to distinguish two phases in the progress of the disease: a *determinative* phase, during which the interaction of the processes which determine the degree of tolerance of the host and the pathogenicity of the fungus establish *those specific conditions of vascular invasion which are pre-requisite for the development of a specific syndrome*; and an *expressive* phase in which the continued activity of the fungus, more or less limited by the conditions established in the determinative phase, results in the development of visible symptoms in the aerial parts of the plant.

Any toxigenic activities of the fungus in the aerial parts thus contribute to the development of the expressive phase and are therefore, in a sense, irrelevant to the elucidation of the mechanisms of host tolerance and fungal pathogenicity. However, the possibility cannot be excluded that toxic substances implicated in the development of leaf symptoms might also play

a part in the establishment of root infection during the determinative phase; it is in this context that studies of the action of the pathogen's metabolites might most profitably be continued. It is of interest to note that Winstead & Walker (1954*b*) have shown that culture filtrates from two races of *Fusarium oxysporum* f. *conglutinans* and from one of *F. oxysporum* f. *vasinfectum*, applied to the roots, induced wilt symptoms only in the *appropriate* susceptible hosts. It was suggested that the organisms produce substances of low molecular weight having a specific effect on susceptible varieties, and possibly in some way aiding the establishment of infection in these varieties.

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EXPLANATION OF PLATE 17

Fig. 1. Leaf showing marginal and interveinal necrosis typical of the acute form of wilt.
Fig. 2. Leaf showing sectorial necrosis characteristic of the mild form of wilt.
Fig. 3. Symptoms induced in a hop leaf by treatment of the shoot with a culture filtrate of *Verticillium albo-atrum*.
Fig. 4. Atypical wilt symptoms developed by an infected plant under glasshouse conditions.

(*Accepted for publication* 5 *November* 1956)

REVIEWS

The Genus Achlya: *Morphology and Taxonomy.* By TERRY W. JOHNSON, Jr. (Ann Arbor: The University of Michigan Press; London: Oxford University Press, 1956.) Pp. xv + 188, 22 plates. Price: 36*s*.

Since the publication of Coker's classic, *The Saprolegniaceae*, in 1923 there has been no monographic treatment of the genus *Achlya*. In these days the thought of a monograph conjures up the heart-warming vision of a mine of essential information, viz. a chronological documented list of all the epithets used under the generic name, the original diagnoses (or descriptions) and figures, and then a critical survey based on type material. The present monograph falls short of this ideal in that it attempts only the last item.

A short introductory section (18 pp.) gives a brief historical résumé, some notes on the occurrence, collection, isolation, and culture of these water moulds and their preservation in culture, and a discussion of taxonomic criteria and terminology. Then follows a systematic treatment of the thirty-five species, one variety, and two unnamed taxa considered by the author to be good taxonomic units. Under each is given a list of synonyms, a full description compiled from the material examined (which is listed), recorded collections, and a brief discussion. For the most part the descriptions were made from living material in pure culture, but only eleven were based on type specimens. The twenty-one plates depicting, somewhat diagrammatically, these thirty-eight taxa are gathered together at the end of the text. For a book which is essentially for 'running down' unknown entities, the most convenient place for 'pictures' is side by side with the descriptions.

The next section deals (in the same way as the preceding) with nine taxa (eight species and one variety) of doubtful affinities; two are invalid and the author does not propose to validate them; for the remainder, material was not available for study and the determination of their systematic position was not considered possible from the published descriptions. Finally there are notes on seventeen excluded taxa.

The author is to be congratulated on his conservative approach to the genus; there is only one nova (the raising of *A. americana* var. *cambrica* to specific rank), and fewer valid taxa are accepted than have been recognized by other students of the genus. Three of Coker's subgenera, *Achlya* (formerly *Euachlya*), *Centroachlya*, and *Subcentrica*, are retained, each with a key to the species.

The monograph will be a valuable source book for students making their first acquaintance with the genus, and for this purpose a glossary would have been useful, indeed is perhaps essential. It is regretted that the term androgynous, rejected fourteen years ago as unsatisfactory in the genus *Pythium*, should have been retained and used in a sense different from that of De Bary for an antheridial relationship correctly termed monoclinous, which is here accorded a different definition.

The book is well produced and reasonably priced. There is a useful bibliography of over 12 pages listing the literature up to 1952.

GRACE M. WATERHOUSE

The Physiology of Reproduction in Fungi. By LILIAN E. HAWKER. (Cambridge Monographs in Experimental Biology No. 6. Cambridge University Press, 1957). Pp. 128, 5 text-figures. Price: 15*s*.

In this book Dr Hawker has bravely essayed the first full-length synopsis of a very diffuse and inexact literature. As she knows, recent studies make it increasingly clear that physiological tolerances and requirements for growth, for the initiation of reproduction and for the subsequent multiple-stage processes of fruit-body development, are not identical, yet perhaps a majority of papers have been concerned only with gross fungus responses such as fruit-body or spore numbers.

Most of the subject-matter is concentrated in chapters 3 to 6. To these is added an introduction defining types of fungus reproduction and the biological functions of spores. Further, there is a well-conceived chapter on morphological and anatomical aspects of

reproduction. The understanding of the structural development of fruit-bodies, as for example whether cell-division or extension growth predominates, is truly described as an essential preliminary to the analysis of form factors. It is more difficult to justify the inclusion of a final chapter discussing reproduction in the natural habitat as there is no work upon which this may be based which is critical even by ecologists' standards.

Vegetative reproduction is conveniently discussed in a separate chapter although this is justifiable more on morphological than physiological grounds, as such generalizations as emerge are also applicable to many cases of asexual and sexual reproduction. A criticism which might be made here and perhaps at one or two other points in the book is that the author has shown a reluctance to go into detail in cases where it might have been profitable. Thus in this section an account of Nickerson's evidence for the probable role of sulphhydryl groups in the physiology of cell division in *Candida* is lacking.

Chapter 4 is concerned with the action of temperature, water (especially atmospheric), 'aeration', hydrogen-ion concentration of the medium, mechanical injury and light upon reproduction. It concludes with a discussion of the interaction of factors, which it is shown may suggest hypotheses concerning the mechanisms of fungus response. Readers may feel that the treatment of the light factor is rather slight in view of its widespread importance. The account would certainly have been enriched by mention of the work of German authors, especially Borriss, on the *Coprinus* sporophore.

Chapters 5 and 6 are in many ways excellent. The first gives an extremely able summary of knowledge of fungus nutrition in relation to reproduction. Not least it will be welcomed as containing a good account of the author's own distinguished studies upon the pattern of fruiting in ascomycetes in relation to carbon sources and organic growth factors. Also, and this is perhaps the most interesting section of the whole book, it ends with a treatment of metabolism and the reproductive phase. The views expressed here, although controversial, will be read with eagerness by all interested in the future of the subject. Briefly the case is argued for (1) an enhanced metabolic rate at the onset of reproduction (especially sexual), and (2) the occurrence of metabolic shunts concerned uniquely with reproduction. The section would have been improved by a more complete and critical account of Cantino's investigation of the changes associated with the production of the dark-walled resting-sporangium in *Blastocladiella emersoni*. His demonstration that when this happens there is a breakdown of the tricarboxylic acid cycle with the accumulation of α-ketoglutarate and its precursors and a de novo synthesis of melanin (which is α-ketoglutarate dependant) is a brilliant instance—the first—of the way in which fruiting studies may go in the future. In chapter 6 an outline of fungus heterothallism introduces a readable account of much of the best fungus physiological work we have, upon the initiation and co-ordination of sexual processes by extrahyphal chemical substances. Work on intracellular biochemical differences between the sexes, especially in pigment content, is considered.

Like all Dr Hawker's publications this book is fluently written. It represents a conservative and timely account of a subject just beginning to expand. With its literature list of 360 references it will be a valuable guide to research workers. The mode of presentation—a succinct review of results—makes it, in the main, not a text for undergraduates.

B. E. PLUNKETT

Biology and Control of the Smut Fungi. By G. W. Fischer and C. S. Holton. (Ronald Press Company, New York, 1957.) Pp. x+622, 107 text-figures. Price: $10.00.

In his preface to the *Manual of North American Smut Fungi*, 1953, Prof. Fischer promised a later volume dealing with the biology of this group of organisms. The present book is the outcome, this time under the joint authorship of himself and C. S. Holton, a sufficient guarantee of authority since it would be hard to find two workers better qualified to undertake this particular task.

An introductory chapter includes a key to genera and species and states the authors' view that the Ustilaginales is best regarded as a single family. Their reasons for this are the largely practical ones of the difficulty of applying criteria based on spore germination to a group of organisms that are notoriously unpredictable in this respect and which, in any case, cannot be used on dead material. This approach may not be acceptable to all

but will at least command the sympathy of those who are familiar with the often capricious behaviour of smut fungi. A most useful feature of this first chapter is a host family register for the Ustilaginales in which the families are arranged in alphabetical order and their smuts listed. A reference for each smut is given but it is a pity that the actual host plants (genera and species) are not named.

A short review cannot do justice to the subject-matter on all aspects of the biology, parasitism and control dealt with in the thirteen chapters in the book. The records and literature from all parts of the world are considered, and the authors are to be commended on the thoroughness with which they have undertaken this very considerable task. Their treatment is no mere recording of published facts but comment based on long practical experience and the inclusion of hitherto unpublished work of their own greatly increases the value and interest of the text.

On reading this book one is impressed once again by the enormous amount of work done on the smuts and yet so much of it is in the form of isolated papers that in the aggregate build up into a mass of information much of which is conflicting. This is well illustrated in the field of smut genetics concerning which the data are considerable and yet, so far, no generally accepted interpretation of the facts has been evolved. This would seem a fruitful subject for a joint attack by mycologists and geneticists, each familiar with the methods of the other, and working together at the same time and place. Cultural characters have frequently been used in genetical studies but, as Thom has so justly pointed out, the culture is not an individual but a population. For smuts it is usually a mass of presumably haploid conidia or hyphae and any interpretation of its character becomes as much a study in micro-ecology as in genetics alone. It is only fair to say that the present authors do not over-emphasize the importance of cultural characters. They are, however, prepared to accept these as legitimate grounds for the separation of physiologic races. Although the term 'physiologic race' and its history is well known the idea behind it, in the minds of pathologists at least, has always been a separation of strains on the grounds of pathogenicity. It might be less confusing if we differentiated and referred to 'parasitic' and 'cultural' races, both of which terms are self descriptive.

A useful survey of control measures concludes the book and it may come as a surprise to British pathologists to learn that wheat bunt is still such a problem in the Pacific North-West. The reason is soil infection, something that we see very little of in this country.

The book is well produced with very few printing errors and any student starting work on the smut fungi will find both information and encouragement in abundance here.

J. H. WESTERN

Crucifer Diseases in Ireland. By ROBERT MCKAY. (Dublin: At the Sign of the Three Candles, 1956.) Pp. viii + 78, 51 figures. Price: 21*s*.

In four previous books Prof. McKay has dealt with the diseases of flax, tomatoes, sugar beet and potatoes. To these he has now added a volume devoted to the diseases of crucifers. This book well maintains the high standards of production set by the publishers of its predecessors in the series. Since the work is directed to farmers and gardeners as well as to agricultural and horticultural advisers it may well be felt that a much wider circulation would have been ensured had the cost been lower, even if this also necessitated a lowering of the publisher's standards.

Consideration is given in this book to twenty-five diseases which are physiological in origin or are caused by bacteria, fungi or viruses or by a few insect pests. The account given of each disease is simple and straightforward, attention being mainly directed to symptoms and control, with a general outline of the life history of the causal organism being presented where this is appropriate. In some instances this approach has resulted in the introduction of generalizations which may not be regarded as being entirely in agreement with the detailed accounts of work carried out on the subject. It is surprising that the effects of temperature on the incidence of club root have been neglected, especially since growers in Ireland may, during winter and spring, successfully raise crops of varieties susceptible to this disease even when the land is heavily contaminated with *Plasmodiophora brassicae* but may be unable to do so under warmer conditions. One other noteworthy omission is the absence of mention of whiptail of crucifers resulting

from calcium deficiency. The number of literature references has been deliberately reduced to a minimum but one finds it difficult to determine what principles have guided the author in the selection he has made.

The book is exceedingly well illustrated with original photographs which are of a uniformly high quality.

JOHN COLHOUN

A Textbook of Plant Virus Diseases. By KENNETH G. SMITH. (J. and A. Churchill Ltd. 1957.) 2nd edition. vii + 652 pp., 94 illustrations. Price 65*s*.

This book must certainly be on the shelves of all plant pathologists and research workers who are concerned with plant viruses or with general phytopathological problems. Its interest for members of this Society is on these rather than on strictly mycological lines. It is a most valuable book of reference, dealing with some 300 viruses and Dr Smith is to be congratulated on the great mass of information he has collated and made readily available. Here is a list of the known plant virus diseases and of the diagnostic features of the causative agent for each. The viruses are listed alphabetically and the author has discarded the system of nomenclature he used in the first edition of the book and has adopted the simpler and more satisfactory system of using the common English names for the disease based on that used in 'The List of Common British Plant Diseases' compiled by the Plant Pathology Committee of this Society. He has, in addition, listed for each the appropriate synonyms under which the virus has already been described. The format is good, the type clear and easily read and the illustrations adequate and helpful in making clear some points of interest. The value of the book for reference purposes would have been greatly enhanced if an index of host plants with the virus diseases known to affect them had been included.

JOHN CALDWELL

Plant Virus Serology. By R. E. F. MATTHEWS. (Cambridge University Press, 1957.) Pp. xii + 128, 6 plates, 12 text-figures. Price: 27*s*. 6*d*.

Although plant pathologists have been using serological techniques to study plant viruses for more than thirty years, this is the first book devoted to the subject. Detailed descriptions of these techniques could be found in the medical or veterinary literature, but were not always readily available, or indeed comprehensible, to botanists. Moreover, there are some difficulties peculiar to serological work with plant viruses which can be overcome only after bitter experience or by the help or advice of a colleague. The extent of this private interchange of ideas is well illustrated by the amount of information in this book obtained by 'personal communication'.

Dr Matthews provides sufficient information to enable a novice to begin work on a serological problem, although some practical instruction in injection procedure might give the operator more self-confidence and make life less unpleasant for the rabbit. The author rightly places most emphasis on the precipitation reaction which is surely the most convenient and consistent in operation. He does rather less than justice, however, to the micro-precipitation technique commonly used in Europe, which he has apparently confused with the much less sensitive agglutination reaction. Both of these methods are of value when economy of antigen or antiserum is of prime importance. A beginner would do well to gain experience in quantitative serology by using the precipitation end-point method in preference to the more refined technique of recording the time taken for precipitation. Dr Matthews has made considerable use of the latter method himself, but as he says, it is to a certain extent subjective, and few pastimes can be more conducive to spots before the eyes than watching for the start of serological precipitation.

The book is excellently printed and illustrated, and provided with well arranged diagrams and tables. There are about a hundred references to works on medical as well as plant virus serology. It should prove useful to anyone making a start on serology of plant viruses, and perhaps suggests new method of approach to those who already have some practical experience.

One hopes that a review in these *Transactions* may also stimulate mycologists and those interested in plant pathogenic bacteria to read this book and make greater use of the techniques in their own particular work.

J. W. BLENCOWE

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TRANSACTIONS

Vol.	PART 1	2	3	4	5
1	*m*	*m*	6*s.*	*m*	*m*
2	(		68*s.*		)
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4	*m*	*m*	—	—	—
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7	(		40*s.*	)	—
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9	(	20*s.*	)	*m*	*m* —
10	(		40*s.*	)	—
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12	(10*s.*)	*m*	*m*	(10*s.*)	—

Vol.	PART 1	2	3	4
13	*m*	*m*	*m*	*m*
14	(		40*s.*	)
15–16	*m*	*m*	*m*	*m*
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CONTENTS

PAGE

Differential rhizosphere effects of three pea cultivars on physiologic races of *Fusarium oxysporum* f. *pisi*. By E. W. Buxton. (With 5 Text-figures) 305

On *Tripospermum* and *Campylospora*. By C. T. Ingold and Vivienne J. Cox. (With 2 Text-figures) 317

Studies on *Pellicularia filamentosa* (Pat.) Rogers. III. Host penetration and resistance, and strain specialization. By N. T. Flentje. (With 2 Text-figures) . . . 322

Anthracnose of banana (*Gloeosporium musarum* Cke. & Massee) with special reference to latent infection in storage. By Tilottama Chakravarty. (With Plate 11 and 3 Text-figures) 337

Dung as a source of predacious fungi. By A. J. Juniper 346

Phytophthora citricola Sawada (Syn. *P. cactorum* var. *applanata* Chester). By Grace M. Waterhouse. (With Plate 12 and 3 Text-figures) 349

Behaviour of soil fungi in the presence of fungal antagonists. By David Park. (With 2 Text-figures) 358

Wortmannin, an antibiotic produced by *Penicillium wortmanni*. By P. W. Brian, P. J. Curtis, H. G. Hemming and G. L. F. Norris. (With Plate 13) 365

Pulvilloric acid, an antibiotic obtained from cultures of *Penicillium pulvillorum*. By P. W. Brian, P. J. Curtis, H. G. Hemming and G. L. F. Norris . . . 369

Studies on vesicular-arbuscular endophytes. I. A strain of *Pythium ultimum* Trow. in roots of *Allium ursinum* L. and other plants. By Lilian E. Hawker, R. W. Harrison, Valerie O. Nicholls and Angela M. Ham. (With Plate 14 and 4 Text-figures) 375

Distribution of fungi within the decomposing tissues of ryegrass roots. By J. S. Waid. (With Plate 15 and 6 Text-figures) 391

Haustoria of *Phytophthora erythroseptica* in potato tubers affected with pink rot. By R. McKay, J. B. Loughnane, and T. Kavanagh. (With Plate 16) . . . 407

A study of air-borne *Cladosporium*. By R. R. Davies. (With 1 Text-figure) . . 409

The possible significance of toxic metabolites of *Verticillium albo-atrum* in the development of hop wilt symptoms. By P. W. Talboys. (With Plate 17 and 7 Text-figures) 415

Reviews 428

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The standards adopted in the *Transactions* are those outlined in the Royal Society's pamphlet, *General Notes on the Preparation of Scientific Papers* (1950, The Royal Society, Burlington House, London, W. 1, price 2*s*. 6*d*.). An exception is that in the *Transactions* citations of references to literature, although based on the Harvard system, include the full titles of the papers and complete page numbers. Titles of journals are abbreviated in accordance with *A World List of Scientific Periodicals*, 1952.

The scientific names of genera and species are printed in italics and should be underlined in the typescript. The title of the paper, headings, and subheadings may be in capitals, but should not be underlined.

The attention of authors proposing new taxonomic groups is drawn to the need to give adequate descriptions and figures, and to designate the types as required by the *International Code of Botanical Nomenclature*. The description of a new species should be accompanied by a figure, and whenever possible the type specimen should be deposited in one of the national herbaria.

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